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Potential Use of IFN-γ-Induced Expression of IDO as a Local Immunosuppressive Factor to Protect Allogenic Skin Substitute Engraftment

by

Kourosh Sarkhosh

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Experimental Surgery

Department of Surgery

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Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the faculty of Graduate Studies and Research for acceptance, a thesis entitled "Potential Use of IFN-γ-Induced Expression of IDO as a Local Immunosuppressive Factor to Protect Allogenic Skin Substitute Engraftment" submitted by Kourosh Sarkhosh in partial fulfillment of the requirements for the degree of Master of Science in Experimental Surgery.

Dr. Hasan Uludag

September 17, 2003

ABSTRACT

Burn injury is a commonly-occurring incident in the adults and children, causing physical morbidity and mortality. The development of a readily available and non-rejectable skin substitute will therefore be beneficial to prevent local and systemic complications associated with these injuries including bacterial infections and heat and fluid loss. The purpose of this study is therefore to induce immuno-tolerance in allogeneic fibroblasts of our biological skin substitute by inducing their IDO expression as a local immunosuppressive factor by IFN-γ treatment.

Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme possessing immunosuppressive properties. IFN-γ is a pleotropic cytokine and inducer of IDO expression in fibroblasts. We have treated fibroblasts with IFN-γ and developed an IDO-expressing skin substitute made up of fibroblasts populated in collagen gel (FPCG). Expression of IDO was inducible in fibroblasts and keratinocytes in a time- and dose-dependant manner. IDO expression didn't alter fibroblast migration and proliferation abilities. Expression of IDO reduced in fibroblasts following IFN-γ removal; however, we demonstrated that by conjugating IFN-γ with a temperature-sensitive polymer, expression of IDO will be prolonged in FPCG. Finally, we showed that expression of IDO in fibroblasts suppresses peripheral blood mononuclear cell (PBMC) proliferation in a co-culture system.

In conclusion, we have developed a biological allogeneic skin substitute of fibroblasts embedded in the collagen gel and expression of IDO in fibroblasts was shown to suppress proliferation of immune cells in a co-culture system.

Dedication

To my family, friends and those whom I hold dear

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LIST OF ABBREVIATIONS

DMEM	dulbecco's modified eagle medium
FBS	fetal bovine serum
FPCG	fibroblasts populated in collagen gel
GITC	guanidium isothiocyanate
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon-gamma
JAK	janus kinase
LCST	lower critical solution temperature
STAT	signal transducer and activator of transcription
MRNA	messenger ribonucleic acid
NASI	N-acryloxysuccinimide
NiPAM	N-isopropylacrylamide
NK	natural killer
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered soluction
PCR	polymerase chain reaction
RT	reverse transcription

CHAPTER ONE:

GENERAL INTRODUCTION

Introduction

Extensive skin loss from a variety of conditions such as severe thermal injury is associated with significant functional morbidity and mortality (Cairns et al., 1993). In recent years, however, the overall mortality rate has improved for patients suffering from burns due in part to the significant biotechnological advancements in skin replacement for wound closure (Cairns et al., 1993).

In vitro cultivation of keratinocytes with the support of a feeder layer of lethally irradiated 3T3-cells was initially introduced by Rheinwald and Green (1975). Although sheets of autologous keratinocytes are currently used in some burn centers to treat patients with large thermal injury (Morthenn et al., 1982; Thivolet et al., 1986), this has not been a routine procedure for several reasons: 1) sheets of keratinocytes prepared from layers of cultured keratinocytes without matrix are very fragile and difficult to cultivate, 2) the rate of graft-take is relatively low (50-60%), 3) patients with large injuries do not have enough uninjured skin to be used for cell culture, 4) leaving the burn wound open for 3-6 weeks until cells become available increases the risk of infections, heat or fluid loss, and finally, 5) generating another wound increases the risk of developing hypertrophic scarring. Considering that all these are pervasive medical problems with far-reaching clinical and economic implications, utilizing an allogeneic and readily available skin substitute would be beneficial.

Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme that converts tryptophan into N-formylkynurenine. Since tryptophan is the least available essential amino acid in the body and highly proliferating cells such as immune cells need it to proliferate, IDO-induced tryptophan depletion plays an important role in physiological

conditions such as the suppression of immune cell responses *in vivo* and *in vitro* (Mellor et al., 2002). It also prevents maternal T-cell mediated rejection of allogeneic fetuses (Munn et al., 1998). Therefore, we hypothesize that IDO expression in allogeneic fibroblasts of our skin substitute can also generate a tryptophan-deficient environment and locally suppress proliferation of immune cells. This could protect the allograft from the infiltrating immune cells and prolong its survival.

This thesis can be summarized into introduction, three major experimental areas and conclusion. First, we describe the effect of IFN-γ on expression of indoleamine 2,3-dioxygenase in the fibroblast-populated collagen gel (FPCG). Expression of IDO is measured in fibroblasts by detecting its mRNA and measuring levels of kynurenine as an indication of IDO enzyme activity. Subsequently, the effect of IFN-γ is studied in fibroblasts and keratinocytes cultured in a monolayer. The immunosuppressive property of IDO is studied on proliferation of allogeneic immune cells. Finally, the role of temperature-sensitive polymers on prolonging the effect of IFN-γ is investigated on fibroblasts populated in collagen gel (FPCG). Immunosuppressive effect of IDO expression in FPCG is then studied in PBMC co-cultured with fibroblasts treated with IFN-γ conjugated with the temperature-sensitive polymer.

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CHAPTER TWO:

LITERATURE REVIEW

Introduction

Burn injury is considered one of the major health hazards for adults and children (Linares et al, 1990). The annual number of burn incidents is reported around 1.25 million in the United States (Brigham et al, 1996), from which 60,000 to 80,000 people get hospitalized (Boyce et al, 2002). The most commonly affected groups are children between 2-4 and young adults between 17-25 years of age. Due to the limited availability of autologous skin in these young patients, an alternative approach of utilizing an allogeneic skin substitute could be beneficial. This biological artificial skin would aim to serve as temporary wound coverage to prevent heat and fluid loss and other arising local or systemic complications. It will also be readily available upon use, thereby reducing patients' hospitalization time. However, similar to any other kind of transplantation, this skin substitute should be made non-rejectable. Therefore, our purpose is to utilize enzyme indoleamine 2,3-dioxygenase as a local immunosuppressive factor to protect our allograft from the immune cell-mediated rejection.

Structure and Function of the Skin

Skin is the largest organ in our body. It has various protective (UV light absorption, mechanical, barrier), perceptive (touch, pain, temperature) and regulatory (thermal, excretory) functions. Skin is composed of two different layers of epidermis and dermis, which are separated by a basement membrane. Dermis and epidermis are attached to each other mainly via collagen types IV and VII forming a complex binding. Failure of this binding causes severe morbidity (Fine et al, 1993).

The epidermis forms about five percent of the skin thickness. The major cell types of epidermis are keratinocytes. Epidermis itself is subdivided into different layers such as strata basale, spinosum, granulosom and corneum (Sheridan et al, 1999). Stratum corneum is the outermost layer of the epidermis and prevents the body from desiccation (Wertz, 2000). Other cell types also reside within the epidermis. Langerhans cells have a dendritic nature and are considered as the skin antigen presenting cells (APCs). They play an important role in allograft rejection and specific T cell responses (Hogan et al, 1995). There are no blood vessels in the epidermis and therefore, the nutrients are absorbed by simple diffusion from the underlying dermis.

Dermis makes up the rest (ninety five percent) of the skin thickness. Fibroblasts are the main cellular constituent of dermis and are embedded in an extracellular matrix made up mainly of collagen, elastin and glycosaminoglycans. The vascular anatomy of skin resides in the dermis and consists of reticular (deep) plexus and papillary (superficial) plexus. The papillary layer is entangled with capillaries and forms dermal papillae that project into epidermis. The connective tissue fibers are more densely woven in the reticular layer than in the papillary layer. Elastin is also more interlaced in the reticular layer. Skin glands also reside in dermis which are either sweat (sudoriferous) or sebaceous glands.

Physiology of the Wound Healing

Wound healing is a dynamic process which involves interaction between many different kinds of cells. It can be divided into three different phases: inflammation,

proliferation and remodeling. These three phases usually overlap with each other and therefore delay in any of them will cause a delay in the whole process of healing.

Inflammation: This phase starts almost immediately after the injury to maintain body homeostasis by forming a blood clot. This formation of blot clot would serve as a scaffold for fibroblasts, neutrophils, monocytes and endothelial cell migration. Disruption of blood vessels also causes diffusion of various blood constituents such as platelets. Platelets, in addition to their primary role of forming blot clot, also secrete platelet-derived growth factor (PDGF) which serves as an important mediator in wound healing.

Another important role of the inflammatory phase is to clean the wounded area of bacteria, foreign particles and other cell debris. Neutrophils are the first cells that migrate into the wounds and their migration is stimulated by several factors such as TNF-α, TGF-β and IL-1. Upregulation of adhesion molecules would facilitate this migration (Smith et al, 1988). Other types of immune cells such as monocytes also infiltrate to the wounded area in response to chemoattractants such as monocyte chemoattractant protein 1 (Singer et al, 1999). Macrophage activation also induces nitric oxide production, which has antimicrobial functions (Witte et al, 1997). Activation of macrophages is also essential for matrix synthesis, angiogenesis and debridement (Polverini et al, 1977). Several important cytokines are secreted by macrophages and monocytes, such as TGF-alpha, interleukin-1 and insulin-like growth factor I (Rappolee et al, 1988). Studies in macrophage-depleted animals showed a defective wound repair (Leibovich et al, 1975) which suggests an important role of these cells in the wound repair process.

Proliferation and matrix deposition: This phase involves new tissue formation in the skin and blood vessels, as well as formation of the granulation tissue. Fibroblasts and endothelial cells are the major proliferative cells in this phase (Witte et al, 1997). Keratinocytes also play an important role in covering the wound to prevent fluid loss and infection. They do so by migrating into the wound from its surroundings. Epithelial cell movement is facilitated by the dissolution of inter-cellular desmosomes and formation of actin filaments (Golinger et al, 1995). Epidermal cells also interact with extracellular matrix fibronection proteins via their integrin receptors (Clark, 1978). Epidermal cell migration is also facilitated by extracellular matrix degradation due to collagenase expression (Pilcher et al, 1997). Macrophages and keratinocytes are known to be sources of stimuli for epithelial cell proliferation (Ansel et al, 1993).

Fibroblasts also get stimulated due to induction by various cytokines such as platelet-derived growth factor and transforming growth factor beta1. They then start to proliferate and migrate into the wounded area (Singer et al, 1999). Fibroblasts also start producing new extracellular matrix, which contributes to formation of granulation tissue with deposition of molecules such as fibronectin (Greiling et al, 1997) and hyaluronic acid (Toole, 1991).

Angiogenesis also occurs during the proliferative phase. This process is dependent upon stimulation and migration of endothelial cells (Madri, 1996). It is mainly induced by the acidic or basic fibroblast growth factors. Low oxygen tension may also induce angiogenesis (Singer et al, 1999). Keratinocytes are also known to secrete substantial amounts of vascular endothelial growth factors (Brown et al, 1992) which can promote angiogenesis. In addition to these growth factors, appropriate endothelial

receptors must also be expressed to serve as a channel for angiogenesis. Functional fibronection receptors are expressed by the endothelial cells (Clark et al, 1982). The fate of cells playing roles in this phase is not completely understood. However, it is known that neutrophils undergo apoptosis and get phagocytosed by macrophages which are then drained into the local lymph nodes (Belligan et al, 1996).

Contraction and remodeling: Different theories have been proposed on the mechanism of wound contraction. Myofibroblasts to have a role in wound contraction since they possess thick bundles called stress fibers. Stimulation by various growth factors such as TGF-β1 and β2 (Montesano et al, 1988), platelet-derived growth factor (Clark et al, 1989) and fibroblasts anchor to extracellular matrix via their integrin receptors (Schiro et al, 1991) have also been proposed to have roles in wound contraction.

Collagen matrix composition also changes during the wound healing process in a time-dependent manner. At first, matrix is made of mostly fibrin and fibronectin that facilitate cellular migration and proliferation into the wound area. Subsequently, glycosaminoglycans and other proteoglycans are synthesized. Finally, collagen molecule dominates the scar composition (Witte et al, 1997). The extracellular matrix of intact dermis consists mostly of type I and III collagen molecules. There also exists a biochemical variance in the structure of collagen residing in non-wounded and wounded skin. Granulation tissue-derived collagen possesses more hydroxylation and glycosylation of lysine residues (Forrest, 1983).

Skin Substitutes

Skin substitutes are classified into different groups based on their characteristics: temporary or permanent; epidermal, dermal or composite; autogenic or allogeneic; and synthetic or biologic. Independent of these, they should posses certain properties to be considered as ideal such as being able to prevent water-loss and not become hypertrophic. An ideal skin substitute should also possess the functional and physical characteristics similar to its recipient. It should also be non-antigenic, durable and can be applied in a single operation (Sheridan et al, 1999).

Temporary Skin Substitutes

This type of skin substitute offers short-term physiologic wound coverage against bacterial infections and body fluid loss. Some of these temporary skin substitutes are:

Porcine Xenograft: This type of graft has been used for temporary coverage of superficial second degree wounds (Chatterjee, 1978) and shown to be effective. It can adhere to the wound and serve as a pain control. However, it doesn't vascularize and might be a potential source of infection (Sheridan et al, 1999).

Allograft: Application of allogeneic cells in the skin substitutes was initiated by Bell et al (1981), who were able to develop an allogeneic skin substitute by embedding both epidermal and dermal cells within collagen, which showed effectiveness in an athymic mice model (Hansbrough et al, 1994). Dermagraft is an example of dermal substitute containing allogeneic fibroblasts embedded within a polymer scaffold made up of polyglycolic acid (polyglactin-910). This development seems to overcome the problems seen in collagen embedded cells such as vascularisation and excessive

collagenase activity (Hansbrough et al, 1994). However, despite promoting re-epithelialisation and ingrowth of vascular tissue, Dermagraft doesn't close the wounds. Therefore, it is mainly used in diabetic foot ulcers rather than burn patients (Naughton et al, 1997). Furthermore, viability of cryopreseved fibroblasts is another issue as they might show poor performance if their level of cytokine production is not enough (Mansbridge et al, 1998).

Permanent Skin Substitutes

Epidermal component: Utilization of this type of skin substitute was expanded after Rheinwald and Green (1975) showed that growth factors and irradiated murine 3T3 fibroblasts, if combined, are able to support human keratinocytes cell cultures on plastic. They were, therefore, able to expand epithelial cells obtained from small skin samples. There exist several types of products that can be utilized as permanent wound coverage. These results enabled O'Connor et al (1981) to use keratinocytes culture as skin grafts for the first time. This method was also used in patients with large wounds (over 95 percent TBSA) and shown to be life-saving (Pittelkow et al, 1986). However, due to absence of dermal component, their durability and engraftment were suboptimal. Cultured autologous kertinocytes can also be applied to the burn wounds (Gallico et al, 1984). These types of products are available commercially (e.g. Epicel). The disadvantages are their fragility, expense and a relatively long time required for their preparation (Jones et al, 2002).

Dermal component: Research in this area strives for a graft that resembles the live human dermis, providing three-dimensional properties of the collagen fibers. In

order for these dermal substitutes to be useful in clinical experiments, they should fulfill certain criteria. They first should be physically strong. Also, fibroblasts within these collagen matrices should remain viable. These types of skin can be either acellular or cellular. Alloderm is an example of acellular graft. It is made from cadaveric skin from which epidermal layer has been removed. Cellular components of dermis have been removed to reduce antigenicity. Alloderm® can serve as a template for dermal regeneration. But, it has little barrier function (Jones et al, 2002). Integra™ initially proposed by Yannas et al (1980), is a cell-free synthetic dermal skin substitute. It is made up of cross-linked bovine collagen and glycosaminoglycan covered with a silicone layer. The dermal pore size has been adjusted to allow fibroblast and endothelial migration and proliferation. Silicone layer is to be replaced with autologous keratinocytes, and therefore, requires a two-stage procedure. Researchers are also trying to incorporate antibiotics to the dermal component to reduce rate of infection (Matsuda et al, 1991). Reported results on Intergra have shown successful engraftment rates and no severe hypertrophic scar formation (Sheridan et al, 1990). Integra™ also offers improved elasticity (Jones et al, 2002).

Composite substitute: These types of grafts contain both epidermal and dermal layers. Apligraft is an example of this type of artificial skin, which is made according to method originally described by Bell et al (1981). It is made up of allogeneic fibroblasts and keratinocytes and is the most sophisticated commercially available skin substitute. The dermal component is comprised of combined mixture of human fibroblasts and type I collagen. Keratinocytes will be seeded onto dermal equivalent after it is cast on a filter disk. Clinical studies have shown promising results in terms of wound closure and lack

of rejection (Sabolinski et al, 1996; Falanga et al, 1998). However, this type of skin has a relatively short shelf-life (about 5 days) and is immunogenic.

Interferon-γ

Interferons are groups of cytokines with similar biological activity and homology. In humans, they are divided into two classes: type I and type II. Type I interferon includes IFN-α, IFN-β and IFN-ω, and type II includes IFN-γ. IFN-γ was discovered 30 years ago through its antiviral activities (Wheelock, 1965). It is produced mainly by activated T helper (Th1) (Mosmann et al, 1989), natural killer (NK) (Perussia, 1991) and CD8+ cytotoxic cells (Sad et al, 1995). IFN-γ exists as a noncovalent 34-kDa homodimer in its biologically active form (Greenlund et al, 1992).

Interferon γ signal transduction pathway: IFN-γ receptor is ubiquitously expressed on all nucleated cells (Farrar et al, 1993; Valente et al, 1992). It is composed of a 90-kDa α-chain with ligand-binding properties and a 314 amino acid β-chain which involves primarily in signaling. The α and β chains of IFN-γ receptor are associated with JAK1 and JAK2, respectively. Signal transduction initiates with binding of IFN-γ to the α receptor which induces α-chain dimerization (Fountoulakis et al 1992). This is followed by association of two β-chains with the α-chains, leading to transphosphorylation and activation of JAKs. The activated JAKs, consequently, phosphorylate both α-chains at 440 tyrosine residues, creating a binding site for cytosolic STAT1α (Greenlund et al, 1994). STAT1α then gets phosphorylated at position tyrosine 701, which results its dissociation from the receptor and creation of STAT1α-homodimers (gamma-interferon activation factor (GAF)) (Greenlund et al, 1995). GAF

then translocates to the nucleus where it binds to specific DNA sequence of gamma activated site (GAS) in the promoter region of the target gene and starts its transcription. This leads to a primary IFN-γ response. Some primary response genes are themselves transcription factors, which may lead to induction of other secondary responses. Cellular response to IFN-γ is due to presence of two sequences in the 5' enhancer region, an ISRE sequence and a complex enhancer (Enhancer A) adjacent to it (Singer et al, 1990). Following signaling, IFN-γ-receptor complex dissociates and gets internalized (Farrar et al, 1993). There exist 2400 high-affinity IFN-γ receptors on each human fibroblast (Anderson et al, 1983).

Biological functions of IFN-γ: Interferon-γ exerts various biological functions. It elevates activity of various enzymes such as 2', 5'-oligoadenylate synthetase, P1 kinase, GTP cyclohydrolase and tryptophanyl-tRNA synthetase (Kadoya et al, 1992). In terms of immuno-regulatory functions, IFN-γ upregulates expression of MHC molecules (Revel et al, 1986) which increases the cellular level of antigen presentation and the phagocytotic abilities of macrophages (Murray et al, 1988). IFN-γ also has inhibitory effects on the cell growth (Trinchieri et al, 1985). It possesses antiviral ability (Samuel, 1991) which was one of the first observed effects of IFN-γ and characteristic to name these groups of cytokines (Isaacs et al, 1957). Administration of IFN-γ can precipitate acute rejection in animals previously rendered tolerant to donor antigens (Bugeon et al, 1992). Interferon-γ induces expression of various enzymes such as tryptophanyl-tRNA synthetase (Rubin et al, 1991), 2',5'-oligoadenylate synthetase (Bonnevie-Nielsen et al, 1991), GTP cyclohydrolase I (Werner et al, 1989) and indoleamine 2,3-dioxygenase (IDO) (Takikawa et al, 1988). Induction of IDO is specific to IFN-γ, but not to interferon

α or β (Takikawa et al, 1988) and therefore, this cytokine has been used to induce IDO expression.

Indoleamine 2,3-Dioxygenase

Indoleamine 2,3-dioxygenase (IDO) is a monomeric enzyme which catalyzes the conversion of tryptophan into N-formyl kynurenine in a rate-limiting step reaction. N-formyl kynurenine is then constitutively converted to kynurenine by the enzyme kynurenine formamidase (Takikawa et al, 1988). Tryptophan is the least available essential amino acid in the body. Near total oxidation of the culture medium tryptophan to kynurenine happens between 48-72 hours (Takikawa et al, 1988). From 22 different amino acids tested, IDO was able to only reduce the tryptophan level significantly (by 63 percent) (Byrne et al, 1986). IDO was first isolated by Higuchi et al (1963) and was further purified by Shimizu et al (1978). IDO is ubiquitously distributed in different mammalian organs such as spleen and brain (Hayaishi et al, 1975), but is present at relatively higher levels in the lung, small intestine and placenta (Yamazaki et al, 1985). Human IDO gene is present as a single copy in a haploid genome. Its size is 15 kilobase pairs and it consists of ten exons and nine introns (Kadoya et al, 1992). IFN-γ-induced IDO expression produces a major and minor mRNA, with sizes of 1.7 and 2.3 kilobases (kb), respectively (Tone et al, 1990). The IDO translation initiation codon lies 33 nucleotides downstream of the 1.7 kb mRNA. TATA-like sequences are present upstream of each transcription start site (Kadoya et al, 1992). *In vitro* translation of IDO revealed that it codes for a protein with the molecular weight of about 42,000 kDa (Dai and Gupta, 1990). IDO is mostly made up of hydrophobic amino acids (Shimizu et al, 1978).

Biological functions of IDO: The induction of IDO is suggested to be important in the host's cell defense mechanisms against pathological conditions (Hiroaki et al, 1996). Expression of IDO blocks replication of intracellular parasites such as *Toxoplasma gondii* (Pfefferkorn, 1984). IDO is also induced *in vivo* in the allografted tumor cells undergoing rejection (Gresser, 1989). The anti-parasitic and anti-tumor activities of IDO have been shown to be due to the tryptophan deficiency (Taylor et al, 1991) since addition of tryptophan reverses them in a dose- and time dependent manner (Leung et al, 1992). IDO expression has been shown to be occurring in cancer patients, which provides a possible host defense mechanism by depleting levels of tryptophan. This tryptophan deficiency has been shown to reduce the rate of cancer cell proliferation (Yasui et al, 1986).

Role of IDO in preventing allogaft rejection: The immunosuppressive property of IDO is an area recently elucidated. IDO is known to be expressed in human placenta in the contact zone between fetal-derived tissues and mother's immune system (Kamimura et al, 1991). Munn et al (1998) showed that expression of IDO can protect fetuses against mother's immune system. They demonstrated that inhibition of IDO results in rejection of allogeneic (but not syngeneic) fetuses. Expression of IDO has been shown to inhibit T cell responses *in vitro* (Lee et al, 2002) and *in vivo* (Mellor et al, 2002) which suggests a mechanism by which IDO inhibits immune cell responses. Schrocksnadel et al (2003) have recently shown a decreased level of tryptophan in women during pregnancy, which might illustrate the expression of IDO during this period. Immunosuppressive function of IDO is not specific to its protective effects in placental cells. IDO expression by different cell types such as macrophages (Munn et al,

1999), dendritic cells (Hwu et al, 2000) and natural killer cells (Frumento et al, 2002) also has been shown to inhibit T cell responses. Recently, Alexander et al (2002) demonstrated that IDO-transfected pancreatic islets have a prolonged rate of survival. This might suggest a potential role for utilizing IDO to prolong the rate of allograft survival.

Thermoreversible Polymers for Protein Delivery

Polymers are long-chain molecules that consist of repeating subunits or monomers. They can either be natural (such as cellulose or DNA) or synthetic. Thermoreversible polymers are synthetic polymers that possess temperature-dependent solubility. Due to this unique characteristic, these polymers have been used extensively for controlled delivery of drugs (Ron et al, 1998) as well as in biological systems for delivery of anti-thrombogenic agents on the blood-contacting surfaces (Gutowska et al, 1995) and cell transplantation (Shimizu et al, 1996). The lower critical solution temperature (LCST: temperature for soluble ↔ insoluble transition) of these polymers plays an important role for delivery of the therapeutic proteins *in vivo* and is below the physiological temperature. This is a temperature at which opposing hydrophobic (due to interaction of $-N-(CH_3)_2$) groups and hydrophilic (mainly H-bonding) forces balance each other out (Uludag et al, 2000). N-isopropylacrylamide (NiPAM) polymers are one group of thermoreversible polymers which have been studied most frequently. Incorporation of succinimide groups in these polymers allows their conjugation to amine groups of proteins (Chen et al, 1990). The LCST of the conjugated polymer is the same as the non-conjugated polymer (Chen et al, 1990). By conjugating proteins to the

polymers, it is possible to introduce the proteins to wound sites and control protein pharmacokinetics (i.e. local concentration as a function of time) of the wound sites. This helps to modulate the biological events involved in wound healing

Graft-Take Process

The skin graft take involves two processes: revascularization and reattachment of the skin graft to the donor site (Barratt et al, 1984). Skin graft healing involves three different phases: serum imbibition (plasmatic circulation), revascularization and organization (Converse et al, 1969). Serum imbibition is the first phase of graft healing process and involves the diffusion of the fibrinogen free serum into the skin graft. Since the graft has no source for its nutrition, it is completely dependent upon this diffusion for survival. Therefore, to facilitate this diffusion, it is important to keep the graft close to the recipient's wound bed. Also, keeping the graft humid improves diffusion. This phase occurs in 24 hours after the skin graft is placed on the wound and lasts for approximately 48 hours. The second phase of graft take healing, phase of re-vascularization, is when blood vessels from the recipient start to grow into the graft (Donato et al, 2000). Presence of the new capillaries in the skin grafts shows an active revascularization phase. The last phase of graft healing, the organization phase, is when the graft has adhered to the recipient bed (Barratt et al, 1984). Therefore, from this point on, the skin graft will function as a native skin. During this phase, the recipient bed will have produced exudate containing leukocytes, erythrocytes and plasma. The fibrinogen present in the exudates is converted to fibrin which facilitates graft adherence to the host bed. Leukocytes also

enter the graft and are present there until blood circulation is established (Rudolph et al, 1973).

Allorecognition (Direct and indirect recognition)

There are two different sets of antigen presenting cells (APC) available: those that are from the donor and those that are from the recipient. Stimulation of T cells by the donor APC is called direct recognition, whereas the T cell stimulation by the recipient's APC is called indirect recognition. Both these processes play important roles in allograft rejection, but which of these two pathways is more important is still controversial. There is some evidence; however, suggesting that the direct recognition is the major pathway involved in rejection since depletion of donor APCs can extend graft survival and direct recognition leads to a stronger mixed lymphocyte reaction (Lafferty et al, 1976). There are two theories suggesting the direct allorecognition mechanism: high determinant density theory and a multiple binary complex theory. The high determinant theory suggests that the T cells recognize the exposed polymorphic residues on the allogeneic MHC molecules. Therefore, the nature of bound peptide is of a secondary importance in this model (Bevan, 1984). The evidence for this model comes from the fact that contact regions between MHC complexes and T cell receptors affect the T cell response (Ajitkumar et al, 1988). Furthermore, synthetic peptide with the same sequences as MHC TCR-binding sites can block the alloresponses (Parham et al, 1987). The second model, the multiple binary complex theory states the anti-MHC alloresponses of the T cells have specificity for peptide/MHC complexes (Sherman, 1993). Since, the peptide sequences of the allogeneic MHC molecules are considerably different from the self-MHC

molecules; therefore each peptide sequence can induce a variety of T cell activations. Evidence for this model comes from the fact that several $CD8^+$ T cells recognize specific peptide/MHC complexes (Smith et al, 1997). Also, the mutant cell line (T2-I-A^b) which is unable to process peptides cannot stimulate alloreponse (Weber et al, 1995). Allorejection seems to be heterogeneous and both above mechanisms seem to be responsible for the overall reponse.

The other important pathway in the alloresponse involves processing of the allogeneic molecules and presenting them by the self-MHC molecules (indirect pathway). The importance of the indirect recognition has been demonstrated by Sayegh et al (1997) by thymic administration of the foreign peptides including allogeneic MHC sequences and prolonging the rate of graft survival. Steele et al (1996) have also illustrated that B cell IgG production in mice undergoing skin transplantation is dependent on indirect transplantation. On the other hand, there exits other support for the indirect response, since rejection can happen by the indirect pathway alone (skin grafts lacking MHC II reject rapidly) (Lechler et al, 1982). Also, in some cases indirect rejection occurs more rapidly than the direct rejection (Auchincloss et al, 1993).

Alloresponse

Alloresponse or allogeneic response is a term used in transplantation immunology to describe the immunological events occurring as a result of transplanting a graft from donor individual to the recipient within the same species. Alloresponse can be divided into two important components: allorecognition, which includes recognition of foreign proteins by the host's lymphocytes and effector mechanisms, which are the mechanisms

generated as a result of this recognition. T lymphocytes are the major cell types involved in graft rejection, since their depletion results in prolonged graft survival (Hernandez-Fuentes et al, 1999). Furthermore, in contrast to low number of T cells that respond to conventional antigens as many as 10% of them react to alloantigens (Ford et al, 1973).

Major Histocompatibility Complex (MHC)

The molecules that cause the most powerful immune response are called major histocompatibility complex (MHC) molecules. These molecules are encoded by the MHC locus on the short arm of chromosome 6 in humans. The product of a single MHC gene is not a homogeneous population of identical structures, but a variety of different peptides in the peptide binding groove (Hunt et al, 1992). The MHC molecules are divided into two different groups: class I and class II. Class I MHC molecules are human leukocyte antigen (HLA) A, B and C in humans and are expressed by all the nucleated cells. Class II molecules are HLA-DR, -DP and -DQ and are only expressed by the bone marrow-derived antigen-presenting cells (APCs) like B lymphocytes, dendritic cells and macrophages and thymic endothelial cells (Abbas et al, 1995). MHC genes are inherited from parents as haploids and therefore, each individual has two sets of chromosomes one coming from each parent (Abbas et al, 1995). MHC molecules are the most polymorphic genes of humans and therefore, each MHC gene has many different variants. MHC molecules play a role of presenting foreign molecules, so they can be recognized by the T cells. Crystal structure studies of the extracellular portions of MHC molecules have revealed that these molecules have a groove, in a place where a peptide is to be bound

(Kacfam et al, 1982). It has also been shown that a T-cell receptor (TCR) recognizes two chains (α1 and α2) and a peptide molecule in the MHC molecules (Garcia et al, 1996).

Minor Histocompatibility Antigen (mHAg)

Minor histocompatibility antigens (mHAgs) are peptides derived from non-MHC polymorphic protein molecules presented by MHC. They were first discovered by Snell (1948) in mice. Unlike the major histocompatibility complexes that are encoded from the certain area on the chromosomes, mHAgs are encoded from loci widely distributed on the chromosomes. These molecules are called "minor" because they cause a less rapid reaction than the MHC molecules. Their importance in the graft rejection was realized when transplanted skin grafts between individuals with similar MHC molecules and multiple mHAg differences underwent rejection (Loveland et al, 1986).

Effector Mechanisms

Role of $CD4^+$ cells:

$CD4^+$ cells play a major role in the graft rejection process. Their function is essential in determining the type of rejection that follows following antigen recognition. $CD4^+$ T cells can interact with the B cells in the lymphoid organs and generate a maturation of alloreactive antibodies (Clark et al, 1994). Furthermore, they can activate APCs and facilitate the priming of $CD8^+$ cytotoxic cells (Ridge et al, 1998). Finally, they can circulate into the graft site, encounter their specific antigen, and secrete proinflammatory cytokines that can attract and activate macrophages (part of the delayed-type hypersensitivity reaction, DTH). The only cells that act independently of $CD4^+$

cells are natural killer (NK) cells and their role in graft rejection is still uncertain (Manilay et al, 1998). Activated $CD4^+$ cells are able to differentiate into three different effector cells according to their cytokine secretion pattern and cell-surface markers (Hall et al, 2000). Th1 cells are involved in secreting interferon-γ (IFN-γ), interleukin-2 (IL-2), lymphotoxin (tumor necrosis factor-β) and augment cell-mediated effector mechanisms. Recipients deficient in the Th1-type cytokines such as IFN-γ (Konieczny et al, 1996) and IL-12 (Piccoti et al, 1996) fail to prolong allograft survival. On the other hand, Th2 cells are involved in secretion of other cytokines, such as interleukin 4 (IL-4), IL-5, IL-6 and IL-10 and promote humoral and allergic reactions (Romagnani et al, 1991; Sher et al, 1992). There is some evidence that production of Th2 type cytokines can reduce the rate of graft survival (Hall et al, 2000). A Th3 subset also has been distinguished to release transforming growth factor-β (TGF-β) which has a role in tissue preservation (Inobe et al, 1998). Cytokine expression pattern in grafts shows that there is a dominant Th1-type response in early and a dominant Th2-type response in long-term surviving allografts (Hall et al, 2000).

Types of Graft Rejection

Hyperacute rejection (HAR):

This form of rejection is rapid and happens within 48 hours of the graft transplantation. It is known to be due to low and high affinity IgM antibodies. Low affinity IgM antibodies are directed against foreign blood group antigens whereas the high affinity IgM antibodies are directed against HLA class I antigens, as a result of prior blood transfusion, pregnancies or failed allografts. It has been shown that these IgM

antibodies are detectable within three days of the allogeneic transplantation or blood transfusion (Wasowska et al, 1992). The binding of antibodies to their specific antigens will cause activation of clotting and finally will lead to necrosis (Hernandez-Fuentes et al, 1999).

Acute rejection:

This type of rejection is less rapid than hyperacute rejection and takes between 5 days and 3 months post-transplantation. There was a controversy during the 1950s about whether cellular or humoral mechanisms are responsible for this type of rejection. Gorer (1942) showed that antibodies are able to reduce tumor cell proliferation *in vitro*. On the other hand, Mitchison (1953) showed that cells (and not antibodies) were able to transfer immunity to allogeneic tumors. In another interesting experiment, Algire (1957) showed that antibodies are able to suppress tissue rejection. Therefore, the leading role of lymphocytes has been established in this type of graft rejection.

Thesis Aims and Experimental Rationale

At the present time, the treatment of patients with large wounds is not satisfactory. There is a need for a readily available and non-rejectable skin substitute. It is our hypothesis that IDO expression in dermal fibroblasts and epidermal keratinocytes generates a tryptophan-deficient microenvironment in which allogeneic immune cells are unable to proliferate. Therefore, it is possible to treat keratinocytes and collagen-embedded fibroblasts with cytokine IFN-γ and induce their IDO expression. This might be a potential role of IDO and shed light towards generating a non-rejectable and readily available allogeneic skin substitute.

Our specific aims are as follows:

1) To evaluate the efficacy of IFN-γ-induced IDO expression in fibroblasts embedded in collagen gel (FPCG) to suppress proliferation of peripheral blood mononuclear cells (PBMC) in a co-culture system. This would be done by:
    I. Treating different strains of fibroblasts populated in collagen gel (FPCG) lattices with IFN-γ and examine their ability to produce IDO
    II. Measuring levels of kynurenine, trytophan oxidation product, in the fibroblast conditioned medium to determine the bioactivity of IDO
    III. Determining the duration of effect of IDO expression in fibroblasts following the removal of IFN-γ
    IV. Evaluating the migration and proliferation abilities of IDO expressing fibroblasts
    V. Examining the ability of IDO expressing fibroblasts to suppress immune cell proliferation in a co-culture system
2) To investigate the effect of IFN-γ on expression of IDO in fibroblasts and keratinocytes cultured in monolayer and study its effect on the co-cultured allogeneic immune cell proliferation. This would be performed by:
    I. Evaluating the IDO expression in fibroblasts and keratinocytes treated with IFN-γ for different durations and concentrations
    II. Measuring tryptophan oxidation product, kynurenine, in the collected cell culture medium as a measurement of IDO enzyme activity
    III. Examining the ability of IDO expressing fibroblasts to suppress proliferation of PBMC in a co-culture system

3) To evaluate the effect of IFN-γ-polymer conjugate on IDO expression, prolongation of IDO expression in fibroblast populated collagen gel (FPCG), and its effect on PBMC proliferation. Also, to conduct an *in vivo* experiment determining the efficacy of utilizing IFN-γ-polymer conjugated IDO-expressing fibroblasts populated in collagen gel (FPCG) to improve the rate of wound healing. This will be carried out by:

   I. Conjugating IFN-γ to a temperature-sensitive polymer

   II. Evaluating IDO expression in FPCG by treating them with IFN-γ-polymer conjugate

   III. Determine the lasting effect of IDO expression in FPCG treated with IFN-γ-conjugated with polymer to investigate whether utilizing polymer can prolong IDO expression

   IV. Examine the rate of proliferation in PBMC co-cultured with polymer-conjugated IFN-γ

   V. Study the rate of wound closure *in vivo* upon treatment of wound with IDO-expressing skin substitute

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CHAPTER THREE:

PROLIFERATION OF PBMC IS SUPPRESSED BY THE INDOLEAMINE 2,3-DIOXYGENASE EXPRESSION OF IFN-γ-TREATED SKIN CELLS IN A CO-CULTURE SYSTEM *

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ABSTRACT

Indoleamine 2,3-dioxygenase (IDO) is an intracellular tryptophan-oxidizing enzyme possessing various immunosuppressive characteristics. In this study, we report the possible use of this enzyme in an allogeneic skin substitute to suppress the proliferation of immune cells. Human fetal skin fibroblasts and keratinocytes were treated with cytokine interferon-γ (IFN-γ) to induce expression of IDO mRNA and protein. The expression of IDO mRNA was evaluated by Northern analysis. IDO enzyme activity was evaluated by measurement of kynurenine level in the IFN-γ treated and untreated cells. Results of Northern analysis showed a dose-dependent response in expression of IDO mRNA in response to various concentrations of IFN-γ used. Northern blot analysis also demonstrated a time-dependent expression of IDO in response to different durations of IFN-γ treatment. The level of kynurenine measured, as the bioactivity of IDO enzyme, was significantly higher in the IFN-γ treated fibroblasts and keratinocytes, compared to those of controls ($p<0.001$). To illustrate the immunosuppressive effects of IDO on immune cell proliferation, IDO-expressing fibroblasts were co-cultured with human peripheral blood mononuclear cells (PBMC) for a period of 5 days. Results of ^{3}H-thymidine incorporation assay showed a significant reduction in proliferation of PBMC co-cultured with IDO-expressing skin cells, compared to PBMC co-cultured with control (non IDO-expressing) skin cells ($p<0.001$). Furthermore, addition of IDO-inhibitor (1-methyl-D-tryptophan) significantly reversed the immunosuppressive effects of IDO on PBMC proliferation ($p<0.001$). In conclusion, suppression of PBMC proliferation due to IFN-γ-induced IDO-expression in allogeneic human skin cells might shed a new light toward developing a non-rejectable allogeneic skin substitute.

INTRODUCTION

Burn injury continues to be a considerable health hazard for adults and children (Linares et al, 1990). The number of people hospitalized due to burn injury is estimated around 60,000 to 80,000 each year in the United States (Boyce et al, 2002), with the most commonly affected age groups being children between 2-4 and young adults between 17-25 years of age (Ryan et al, 1992). However, the overall mortality rate has been recently improved for patients suffering from burns. This is due in part to the significant biotechnological advancements in skin replacement to facilitate the rate of wound closure (Cairns et al, 1993).

In vitro cultivation of keratinocytes with the support of a feeder layer of irradiated murine 3T3 fibroblasts was initially introduced by Rheinwald and Green (Rheinwald et al, 1975). These investigators were able to grow autogenic keratinocytes to confluency, which was suitable for skin grafting (Gallico et al, 1984). The procedure of expanding skin cells from an uninjured skin, however, is not feasible for many patients, such as those with large dermal injury. In addition, leaving a large wound open, for at least 4-6 weeks until autologous skin cells become available, would increase the risk of infection, fluid and heat loss. This would also increase the chance of developing post-burn dermal fibroproliferative disorders, such as hypertrophic scarring and keloid. Thus, the best solution to overcome these problems stated is to prepare a readily available non-rejectable allogeneic skin substitute which might be used immediately upon need. Here, we therefore designed and conducted a series of experiments to examine the benefit of utilizing indoleamine 2,3-dioxygenase (IDO) as a local immunosuppressive factor expressed by fibroblasts and keratinocytes.

Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme catalyzing the rate-limiting step in conversion of tryptophan, the least available essential amino acid in the body, to kynurenine (Taylor et al, 1991). The antitumour and antiparasitic properties of IDO have been previously demonstrated to be due to tryptophan deficiency (Gresser, 1989). The immunosuppressive property of IDO, however, has been recently explored. Munn et al (1998) have shown that IDO-expressing cells, located at the maternal-fetal interface of human placenta (Kamimura et al, 1991), protect the allogeneic fetuses from mothers' immune system. These IDO-expressing cells are shown to inhibit T cell responses during pregnancy (Mellor et al, 2001). This suggests a novel mechanism by which IDO protects the allogeneic fetuses. Furthermore, the expression of IDO in macrophages is also shown to inhibit proliferation of CD4+ T lymphocytes, CD8+ T lymphocytes and natural killer (NK) cells (Frumento et al, 2002), further signifying the immunosuppressive ability of this enzyme. In addition, IDO has been utilized to prolong islet survival in the insulin-producing pancreatic cells (Alexander et al, 2002), suggesting the feasibility of using IDO to prolong allograft survival. Induction of IDO in skin cells seems to be specific to interferon–γ, but not to interferon α or β (Takikawa et al, 1988).

Interferon gamma (IFN-γ), a pleotropic cytokine secreted by the natural killer (NK) and activated T cells (Boehm et al, 1997), plays an important role in our immune system. IFN-γ-induced IDO expression possesses various anti-parasitic (Dai et al, 1994), anti-viral (Cheney et al, 2002), antibacterial (Daubener et al, 1999) and anti-tumour (Burke et al, 1995) properties. IFN-γ exerts its effects by binding to specific receptors on human fibroblasts (Anderson et al, 1982) and keratinocytes (Koizumi et al, 1990), which will then activate the Jak-STAT signal transduction pathway (Sadir et al, 2000). IDO

expression is shown to be halted in the IFN-γ resistant mutant cell lines (Feng et al, 1989), which may confirm the crucial role of this cytokine in stimulation of IDO expression and production.

Since skin keratinocytes play a critical role as antigen presenting cells in allogeneic skin grafting (Cauza et al, 2002; Schwarz et al, 2002), it would be important to see whether IDO expression protects these cells from the infiltrated immune system. Thus, in the light of new findings on the role of IDO in preventing maternal T-cell mediated rejection of allogeneic fetuses (Mellor et al, 2001; Munn et al, 1998), here we hypothesize that proliferation of allogeneic infiltrated immune cells would be suppressed when co-cultured with IDO-expressing fibroblasts and keratinocytes. In this study, we therefore evaluated the efficacy of IFN-γ-induced IDO expression in skin cells on the proliferation of human peripheral blood mononuclear cells (PBMC) in a co-culture system. The findings of this study revealed that IFN-γ induces the expression of IDO protein and its enzyme activity in the skin fibroblasts and keratinocytes. This in turn generates a tryptophan-deficient microenvironment where human immune cells are unable to proliferate. The findings of this study may, therefore, shed light toward developing a non-rejectable skin substitute to be used as wound coverage.

MATERIALS AND METHODS

Fibroblast and keratinocyte cell culture:

Cultures of human foreskin fibroblasts were established as described previously by Ghahary et al (1997). In brief, punch biopsy samples were prepared from human foreskin fibroblasts. The tissue was collected in DMEM with 10% FBS (GIBCO, Grand

Island, NY), minced into small pieces of less than 0.5 mm in any dimension, washed with sterile medium for six times, and distributed into 60×15 mm Petri culture dishes (Corning Inc., Corning, NY), four pieces per each dish. A sterile glass cover-slip was attached to the dish with a drop of sterile silicone grease to immobilize the tissue fragment. DMEM + Ab (penicillin G sodium 100 U/ml, streptomycin sulfate 100 μg/ml, and amphotericin B 0.25 μg/ml) (3ml) with 10% FBS was added to each dish and incubated at 37°C in a water-jacked humidified incubator in an atmosphere of 5% CO_2. The medium was replaced twice weekly. After 4 weeks of incubation, cells were released from dishes by brief (5 min) treatment with 0.1% trypsin (Life technologies Inc., Gaithersburg, MD) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) in PBS (pH 7.4) and transferred to 75 cm^2 culture flasks (Corning Inc., Corning, NY). Thereafter, once visual confluence was reached, the cells were subcultured 1:6 by trypsinization. Fibroblasts from passages 4 to 7 were used for this study. The procedure of Rheinwald and Green (1975) was used for cultivation of human foreskin keratinocytes using keratinocyte serum-free medium (KSFM) (GIBCO, Grand Island, NY) supplemented with bovine pituitary extract (25 μg/ml) and epidermal growth factor (0.5 ng/ml). Primary cultured keratinocytes at passages 3 to 5 were used for this study.

IFN-γ treatment:

Fibroblasts and keratinocytes were seeded onto six-well plates with 35 mm diameter (ICN Biomedicals Inc., Aurora, OH) in duplicate 7.5×10^5 cells/well [2.5×10^5 cells/ml] overnight. Cells were then treated with 1000 U of IFN-γ/ml for a period of 48

hr, unless stated otherwise. Recombinant human IFN-γ (Sigma Chemical Co., St. Louis, MO) was used for this study.

Northern blot analysis:

Fibroblasts and keratinocytes were then harvested by a brief (5 min) treatment with 0.1% trypsin (Life Technologies Inc., Gaithersburg, MD) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) in PBS (pH 7.4). Cells were pelleted by centrifugation at 1100 rpm for 10 min and then lysed with 500 µl of 4 M guanidium isothiocyanate (GITC) solution. Total RNA from each group was isolated by the guanidium isothiocyanate/CsCl procedure of Chirgwin et al (1979), using phenol:chloroform (1:1), followed by chloroform:isoamyl alcohol (49:1). Total RNA from each individual fibroblast culture was then separated by electrophoresis (10 µg per lane) on a 1% agarose gel containing 2.2 M formaldehyde and was blotted onto a nitrocellulose filter. To control the loading, quantities of 18S ribosomal RNA were compared visually by ethidium bromide fluorescence. The blots were baked for 2 hr at 80°C under vacuum and prehybridized for 4 hr at 45°C in a prehybridization solution. Hybridization was performed at 45°C in the same solution containing cDNA specific for either IDO or 18S cDNA. The cDNA probes were labeled with P-α^{32}-dCTP by nick translation. The filters were washed initially at room temperature with 2×sodium citrate/sodium chloride buffers and 0.1% sodium dodecylsulfate for 1 hr and finally washed for 20 min at 65°C in 0.1×sodium citrate/sodium chloride buffer and 0.1% sodium dodecylsulfate. Autoradiography was performed by exposing Kodak X-Omat film to nitrocellulose filters at -80°C, in the presence of an intensifying screen.

Quantitative analysis of autoradiographs was accomplished by densitometry. The cDNA probe for 18S was obtained from the American Type Culture Collection (Rockville, MD). The IDO cDNA probe was a gift from Dr. J.M. Carlin (Department of Microbiology, Miami University, USA).

RT-PCR analysis:

The cDNA was synthesized from extracted total RNA from fibroblasts and keratinocytes, using oligo (dT) primer and MMLV reverse transcriptase (GIBCO-BRL). Samples were incubated at 42°C for 60 min, and the reaction was then terminated by heating at 70°C for 15 min, followed by rapid chilling on ice. PCR were carried out with IDO primers (sense: 5'-GACTACAAGAAAGAGTACCA-3'; antisense: 5'-TTGGGTTACATTAACCTTCC-3'; with an approximate size of 1200 bp) for 35 cycles. PCR products were separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide. DNA bands were visualized under UV light.

Determination of total kynurenine in the conditioned medium:

The biological activity of IDO was evaluated by measuring the level of kynurenine, the degradative product of tryptophan oxidation, present in the conditioned medium derived from IFN-γ treated fibroblasts and keratinocytes. The conditioned medium related to the same number of cells was first deproteinized by adding 100 μL of acetone to 50 μL of the cell culture medium. Sample was vortexed, cooled on ice and centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant of each sample received 10 μl of 0.25 M $HClO_4$ and IS (4-amino-hippuric acid) solution and the mixture was

incubated at 25°C for 25 min. The mixture was then evaporated to dryness under vacuum. 100 μL of 14% BF_3-propanol of was added to the residue in a screw-capped glass tube and was heated at 90 °C for 2 hr. The solution was again evaporated to dryness under vacuum. The residue was dissolved in 40 μL of 20% MeOH in water and was analyzed by liquid chromatography with electrospray mass spectrometry (LC/EMS), using a Hewlett Packard series 1100 mass selective detector controlled by 1100-MED Chem Station. The mass spectrometer was operated in the positive ion mode. Kynurenine quantitative analysis was performed in the selected ion-monitoring mode.

Peripheral blood mononuclear cell (PBMC) isolation and mixed lymphocyte reaction:

Samples of 25 to 30 ml of human blood were drawn into heparinized tubes from normal individuals. Total PBMC was isolated by density gradient sedimentation on Histopaque 1077 (Sigma, St. Louis, MO) according to the manufacturer's protocol. Briefly, whole blood was layered onto an equal volume of Histopaque and centrifuged at 2000 rpm for 20 min at room temperature. Peripheral mononuclear blood cells were isolated and added to RPMI 1640 + 10% FBS (GIBCO, Grand Island, NY) and pelleted by centrifugation at 2000 rpm for 10 min and were further washed twice in RPMI 1640. For the mixed lymphocyte reaction experiment, fibroblasts were treated with 1000 U of IFN-γ for a period of 48 hr, after which IFN-γ was removed, cells were washed three times with PBS and fresh medium (with no IFN-γ) was replaced. Subsequently, 5×10^4 fibroblasts were plated into twelve-well plates (Costar, Cambridge, MA), irradiated with 2000 units of gamma rays and co-cultured with 2×10^5 of PBMC in RPMI 1640 medium

supplemented with 10% FBS for a period of 5 d. Specificity of IDO action on PBMC proliferation was determined by addition of 800μM IDO inhibitor, 1-methyl-D-tryptophan (Aldrich Chem. Co., Milwaukee, WI), to the IDO-expressing fibroblasts.

^{3}H-thymidine incorporation assay:

To measure the rate of PBMC proliferation in response to exposure to allogeneic fibroblasts, ^{3}H-thymidine proliferation assay was performed on the PBMC co-cultured with fibroblasts. In brief, 2 μCi of ^{3}H-thymidine (PerkinElmer Life Sciences Inc., Boston, MA) was added to each ml of the conditioned medium and incubated for 16 hr. After this period, PBMC were harvested, washed three times with PBS, dissolved in GITC and added to the scintillation fluid (Amersham Corp., Arlington Heights, IL). Radioactive counting was performed using the scintillation counter (Beckman).

Statistical analysis:

Student unpaired two-tailed t-test was used to compare the kynurenine levels and PBMC proliferation between IFN-γ treated and untreated skin fibroblasts and keratinocytes. P-values <0.05 were considered to be significant.

RESULTS

Expression of IDO mRNA in fibroblasts and keratinocytes upon IFN-γ treatment:

To investigate whether IFN-γ treatment induces IDO mRNA expression in skin cells, RT-PCR analysis was performed on the extracted total RNA from IFN-γ untreated and treated fibroblasts and keratinocytes. Figure 1.3, depicts the representative RT-PCR

result showing that IDO mRNA expression is inducible in fibroblasts and keratinocytes upon 48 hr treatment with 1000 U of IFN-γ (Lanes T1 and T2, respectively). However, expression of IDO mRNA was not detectable in the IFN-γ-untreated fibroblasts and keratinocytes (Lanes C1 and C2, respectively).

Dose-dependent expression of IDO mRNA in the skin fibroblasts and keratinocytes upon IFN-γ treatment:

To statistically analyze the pattern of IDO mRNA expression in skin cells upon IFN-γ treatment, Northern blot analysis was performed on the total RNA extracted from IFN-γ-treated fibroblasts and keratinocytes. We initially determined the optimum dose of IFN-γ and time required for maximum cellular response to treatment. Using this optimum dose and timing, the levels of IDO mRNA and enzyme activity were evaluated in different cell strains. The results of IFN-γ dose-response shown in Figures 2.3A and B, depict the pattern of IDO mRNA expression in fibroblasts (Panel A) and keratinocytes (Panel B) treated with either nothing (control), 100, 500, 1000 or 2000 U of IFN-γ. As shown in this figure, both IFN-γ untreated fibroblasts and keratinocytes do not express detectable levels of IDO mRNA; however, upon treatment with 1000 U of IFN-γ, IDO expression was markedly induced in these cells. 18S ribosomal RNA was used as a loading control. The results on Figures 2.3C and D, also illustrate the ratios of IDO mRNA/18S rRNA in either fibroblasts (Panel C) or keratinocytes (Panel D) from the combined densitometry results of three separate experiments. The asterisks denote a significant difference in level of IDO mRNA expression between IFN-γ treated and untreated fibroblasts and keratinocytes (* $p<0.001$).

Time-dependent expression of IDO mRNA in skin fibroblasts and keratinocytes:

To investigate the pattern of IDO mRNA expression in skin cells upon treatment with 1000 U of IFN-γ for different time-intervals, total RNA was extracted from IFN-γ treated and untreated fibroblasts and keratinocytes and subjected to Northern analysis. Figures 3.3A and B, show the pattern of IDO mRNA expression in fibroblasts (Panel A) and keratinocytes (Panel B) upon treatment with 1000 U of IFN-γ for a period of either 0 (Control), 4, 8, 24, 48 or 72 hr. The IDO mRNA expression was markedly induced in human fibroblasts as early of 8 hr and reached to its maximum level within 48 hr of treatment. Similarly, as shown in Figure 3B, the IDO mRNA expression was markedly induced in human keratinocytes following IFN-γ treatment. 18S ribosomal RNA was used as a loading control. The results of Figures 3.3C and D demonstrate the combined densitometry data on ratio of IDO mRNA/18S rRNA expression in either fibroblasts (Panel C) or keratinocytes (Panel D) treated with 1000 U of IFN-γ for different durations obtained of three separate experiments. IDO mRNA expression was markedly induced in different strains of human fibroblasts and keratinocytes as early as 8 and 24 hr for fibroblasts and keratinocytes, respectively. A comparison between these two cell types showed that keratinocytes (Panel D) have a delayed response to IFN-γ treatment compared to that of fibroblasts (Panel C). The p-value of <0.001 for IDO mRNA expression between IFN-γ treated and untreated fibroblasts and keratinocytes is considered to be extremely significant (* symbol).

Increased kynurenine levels in the IFN-γ-treated fibroblasts and keratinocytes:

To measure the biological activity of IDO protein on skin cells, kynurenine levels were measured in the conditioned media collected from IFN-γ untreated (control) and treated fibroblasts and keratinocytes. Figures 4.3A and B, show the kynurenine levels in either fibroblasts (Panel A) or keratinocytes (Panel B) treated with different doses of IFN-γ. Combined data from three separate experiments for each cell types showed that treatment with as low as 1000 U of IFN-γ significantly increases the levels of kynurenine in both fibroblasts and keratinocytes (0.84 ± 0.09 and 1.86 ± 0.3 μg/ml, respectively), compared to that of untreated controls (0.02 ± 0.01 μg/ml and 0.2 ± 0.05 μg/ml, respectively). In accordance to the level of IDO mRNA, which was induced at lower concentrations of IFN-γ in keratinocytes, the enzyme activity (kynurenine levels) seems to be also higher in keratinocytes relative to that shown for fibroblasts, at lower doses of IFN-γ. In a similar experimental setting, the level of kynurenine was also measured as a function of timing of IFN-γ treatment. Figures 4.3C and D, show the kynurenine levels in either fibroblasts (Panel C) or keratinocytes (Panel D) untreated (circles) or treated (squares) with 1000 U of IFN-γ for a period of either 0 (control), 4, 8, 24, 48 or 72 hr. The level of kynurenine production after 48 hr of IFN-γ treatment was significantly higher in both cell types than those of controls (* $p<0.001$, $n=3$). However, minor differences were found between kynurenine levels in the IFN-γ treated skin cells from the results of time- and dose-dependent response experiments, which seem to be due to existing variations among different experiments.

Suppression of proliferation in PBMC co-cultured with IDO-expressing fibroblasts:

To demonstrate the immunosuppressive effects of IDO expression in skin cells on the proliferation of immune cells (PBMC), fibroblasts were treated with IFN-γ to induce IDO expression. To eliminate the effects of IFN-γ on PBMC proliferation, fibroblasts conditioned medium was replaced with fresh medium without IFN-γ and isolated human PBMC were co-cultured with these IDO-expressing skin fibroblasts. The results of ^{3}H-thymidine incorporation assay showed a significant reduction in proliferation of PBMC co-cultured with IDO-expressing fibroblasts, compared with that of PBMC co-cultured with non-IDO expressing fibroblasts (*$p<0.001$). The suppression of immune cell proliferation was specific to IDO expression, since this effect was reversible upon addition of 800 μM of IDO inhibitor 1-methyl-D-tryptophan. The results also showed a significant difference in proliferation of PBMC co-cultured with IDO-expressing fibroblasts, compared with PBMC co-cultured with IDO-expressing fibroblasts in presence of IDO inhibitor (* $p<0.001$) (Figure 5.3).

DISCUSSION

The purpose of this study was to investigate the effect of IDO expression by skin cells on the proliferation of co-cultured peripheral blood mononuclear cells (PBMC). Cytokine IFN-γ was utilized to induce the expression of IDO enzyme in human fibroblasts and keratinocytes. IDO expression was found to be inducible in skin cells upon IFN-γ treatment. Furthermore, this IFN-γ-induced IDO expression occurred in a dose-and time-dependent manner, with both fibroblasts and keratinocytes having an optimal IDO mRNA expression following 48 hr of IFN-γ treatment. The bioactivity of IDO protein on skin cells was determined by measuring level of kynurenine, the

degradation product of tryptophan oxidation. The findings of this study show that IDO-expressing skin cells significantly suppress the immune cell proliferation in their vicinity. This might make IDO a suitable enzyme to be utilized in a non-rejectable allogeneic skin substitute.

Burn injury is a commonly occurring incident in both adults and children.[1] As the culture of skin cells and preparation of autologous skin substitute is not feasible at least within the 4-6 weeks of post injury, development of an allogeneic skin substitute as wound coverage seems to be critical for patient's survival. Since this cultured skin substitute should possess the same functional and structural properties as the normal skin (Balasubramani et al, 2001), both fibroblasts and keratinocytes as two primary cell types in dermis and epidermis were used in this study, respectively. Although, T cell activation and proliferation seems to be responsible for allogeneic graft rejection, here, we evaluated the proliferation of peripheral blood mononuclear cells (PBMC) because the majority of these cells should be T cells.

Here, we established that IFN-γ induces the expression of IDO mRNA expression and that consistent with the enzyme activity by measuring the kynurenine in conditioned medium of treated and untreated cells. Since enzyme IDO oxidizes the least available essential amino acid (tryptophan) to kynurenine (Holmes, 1998), its expression would generate a tryptophan-deficient environment in which high proliferating cells such as immune cells are unable to proliferate and attack the allogeneic skin cells. In fact, the result of mixed lymphocyte reaction assay proved that IDO-expressing skin fibroblasts generated a high kynurenine and low tryptophan level conditioned medium in which activated PBMC are unable to proliferate. This suppression was reversible by an addition

of IDO inhibitor (1-methyl-D-tryptophan) and that demonstrates the specificity of IDO action in reducing immune cell proliferation. This is anticipated to be the same when IDO-expressing epidermal cells are co-cultured with activated allogeneic human PBMC.

Time-response experiment on skin cells on the level of IDO production (by measurement of the levels of kynurenine) in response to various durations of IFN-γ treatment showed that dermal fibroblasts respond faster, relative to epidermal keratinocytes, in producing IDO protein. Although further studies are needed to clarify this point, this might be due to differences in either rate of IDO protein translation, the environment in which the enzyme is active, or both, between fibroblasts and keratinocytes. Interestingly, the opposite happened in the dose-response experiment. In other words, kynurenine levels were significantly increased in keratinocytes at relatively lower doses of IFN-γ than in fibroblasts, suggesting that keratinoytes are more sensitive to lower concentrations of IFN-γ. This phenomenon might be due to a variation in the relative abundance of IFN-γ receptor (IFN-γ R) on the surface of skin fibroblasts and keratinocytes.

Finally, another important concern in utilizing IDO as an immunosuppressive agent is considering its effect on the proliferation of IDO-expressing skin cells. In various experiments performed in our laboratory using IDO adenoviral infected cells, we were able to show that different human cells such as CD4+ immune cells (Jurkat cells) and monocyte cell line respond differently to the tryptophan-deficient environment, according to their degree of proliferation (unpublished data). For instance, CD4+ cells, which have an important function in graft rejection, are very sensitive to low tryptophan concentration, whereas THP-1 monocytes are much less sensitive to low tryptophan

concentration. Skin cells also seem to be resistant to low tryptophan concentration (unpublished data). In fact, we observed that proliferation and migration abilities of skin fibroblasts and keratinocytes are not altered in the IDO-induced tryptophan-deficient environment (data not shown). This means that IDO expression influences the high proliferating immune cells, but skin cells seem to proliferate and migrate normally *in vivo*. The role of IDO-expressing cells of a skin substitute in an *in vivo* setting is under current investigation.

In conclusion, we showed that IFN-γ treatment induces the expression of IDO mRNA and protein in epidermal keratinocytes and dermal fibroblasts *in vitro*. The tryptophan-deficient environment, created by IDO protein, produces a milieu in which stimulated immune cells are unable to proliferate. Although, further studies are required to address different issues which were not raised in this study, this finding just initiates a new approach to utilize the immunosuppressive enzyme, IDO, to generate a non-rejectable allogeneic skin substitute in future.

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FIGURE LEGENDS

Figure 1.3. Fibroblasts and keratinocytes express IDO mRNA upon IFN-γ treatment.

Skin cells were either untreated (control) or treated with 1000 U of IFN-γ for a period of 48 hr. The extracted total RNA was reverstrancribed to its related cDNA and PCR was carried out to amplify the IDO cDNA using primers specific for IDO gene by procedure described in the Materials and Methods. PCR products were run on a 1% agarose gel. Results show that IDO mNRA is inducible in fibroblasts and keratinocytes upon treatment with IFN-γ (Lanes T1 and T2, respectively), whereas the level of IDO mRNA expression is not detectable in both untreated fibroblasts and keratinocytes (Lanes C1 and C2, respectively).

Figure 2.3. Dose-dependent expression of IDO mRNA in the IFN-γ-treated fibroblasts and keratinocytes.

Fibroblasts (Panel A) and keratinocytes (Panel B) were treated with either 0 (control), 100, 500, 1000 or 2000 U of IFN-γ for a period of 48 hr. Total RNA was then extracted and subjected to Northern analysis. Panels A and B, are the representative patterns of IDO mRNA expression in IFN-γ untreated and treated fibroblasts and keratinocytes, respectively. Expression of 18S rRNA was used as a loading control. Data is also shown as densitometry units on the ratio of IDO mRNA/18S rRNA in fibroblasts (Panel C) and keratinocytes (Panel D) (* $p<0.001$, $n=3$).

Figure 3.3. Time-dependent expression of IDO mRNA in IFN-γ-treated fibroblasts and keratinocytes.

Fibroblasts (Panel A) and keratinocytes (Panel B) were treated with 1000 U of IFN-γ for either 0, 4, 8, 24, 48 or 72 hr. Expression of IDO mRNA was then evaluated by Northern blot analysis performed on the extracted total RNA. Panels A and B, depict the representative Northern blots showing the time-dependent pattern of IDO mRNA expression in fibroblasts and keratinocytes, respectively. 18S rRNA is used as a loading control. Panels C and D, are the combined densitometry results of three separate experiments, showing the ratios of IDO mRNA/18S rRNA expression, in the IFN-γ treated fibroblasts and keratinocytes, respectively (* $p<0.001$, $n=3$).

Figure 4.3. Increased kynurenine production in the IFN-γ-induced IDO-expressing fibroblasts and keratinocytes.

Fibroblasts (Panel A) and keratinocytes (Panel B) were treated with either nothing (control), 100, 500, 1000 or 2000 U of IFN-γ for a period of 48 hr. The conditioned medium was then collected and evaluated for kynurenine content, using the procedure described in the Materials and Methods. Panels A and B, are results of the combined data from three separate experiments performed on fibroblasts and keratinocytes, respectively. In a similar experimental condition, skin cells were also treated with 1000 U of IFN-γ for duration of either 0, 4, 8, 24, 48 or 72 hr. Panels C and D, demonstrate the level of kynurenine production expressed IFN-γ untreated (circles) and treated (squares) fibroblasts (Panel C) and keratinocytes (Panel D). Data is shown as the mean +/- SD (* $p<0.001$, $n=3$).

Figure 5.3. Suppression in the proliferation of PBMC co-cultured with IFN-γ-induced IDO expressing fibroblasts.

Fibroblasts were treated with 1000 U of IFN-γ for 48 h, after which IFN-γ containing conditioned medium was removed, cells were washed three times with PBS, and the conditioned medium was replaced with fresh medium with no IFN-γ. IFN-γ-treated and untreated cells were then irradiated with 2000 units of gamma rays and co-cultured in triplicates with isolated human PBMC for 5 days. After this period, 2 μCi of ^{3}H-thymidime was added to each co-culture and incubated in a culture chamber for 16 hr. Floating PBMC were then harvested, washed three times with PBS and incorporation of ^{3}H-thymidime into cellular DNA was evaluated according to the procedure described in Materials and Methods. PBMC were cultured either alone (Column 1), co-cultured with non-IDO expressing fibroblasts (Column 2), or co-cultured with IDO-expressing fibroblasts in absence or presence of 800 μM IDO inhibitor (Columns 3 and 4, respectively). Data represents the mean +/- SD for 4 separate experiments. The asterisk (* $p<0.001$) denotes a significant difference in proliferation of PMBC co-cultured with IDO-expressing fibroblasts, compared to those co-cultured with non IDO-expressing fibroblasts (Columns 2 and 3), as well as a significant difference in proliferation of PMBC co-cultured with IDO-expressing fibroblasts, in absence and presence of IDO inhibitor (Columns 2 and 4).

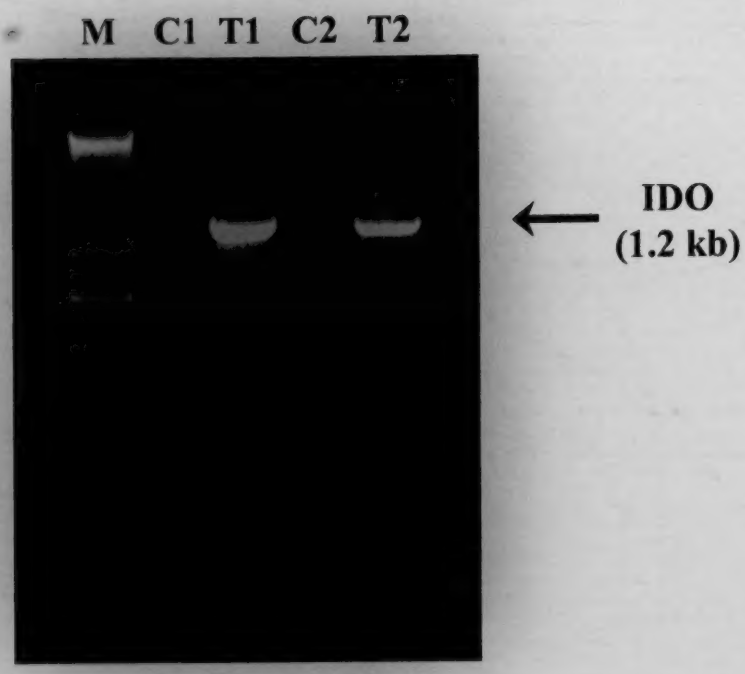


Figure 1.3

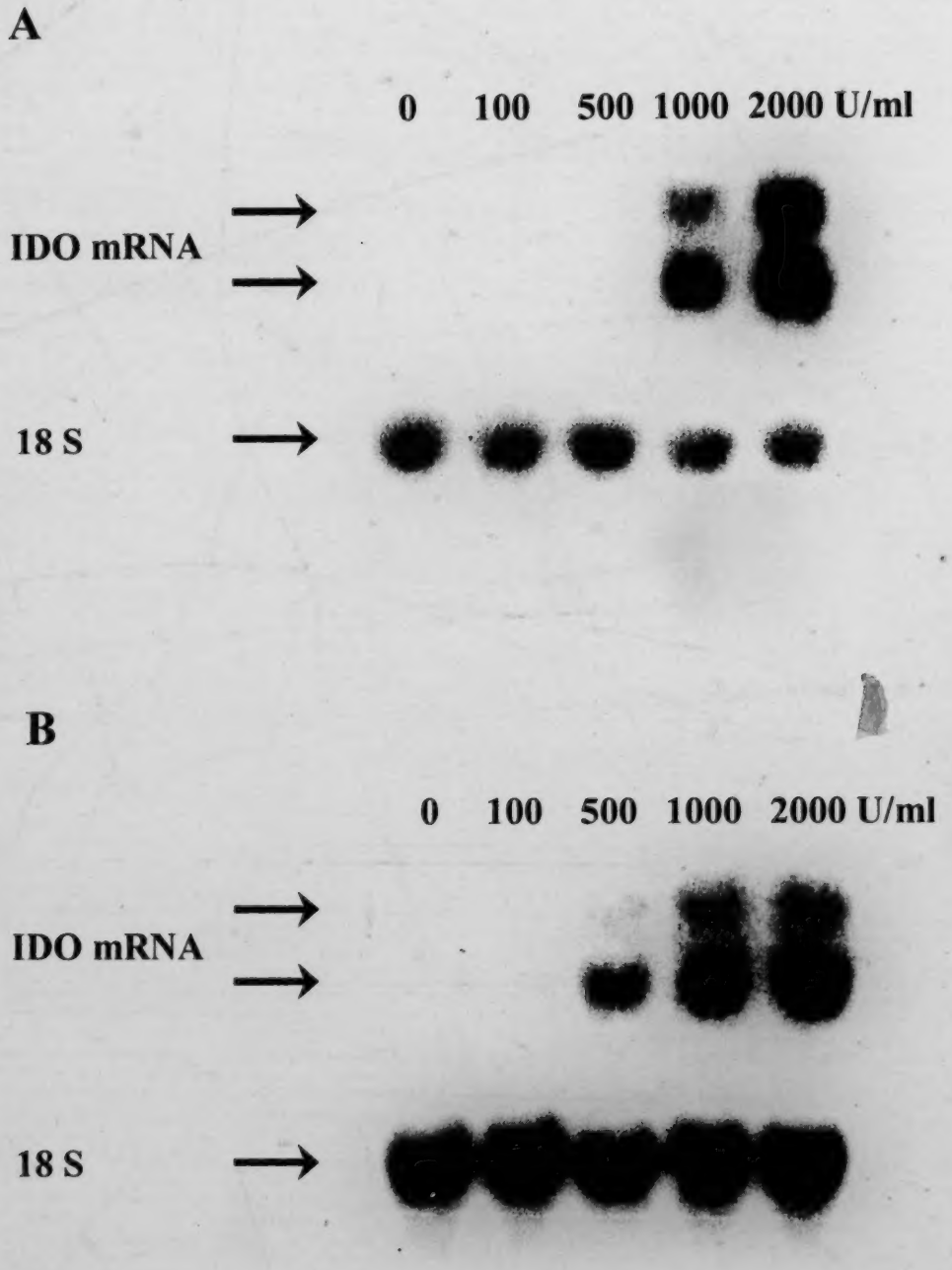
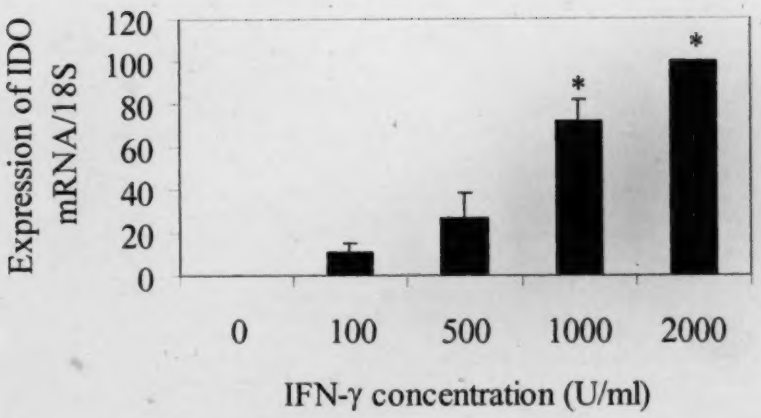

A
0 100 500 1000 2000 U/ml
IDO mRNA
18 S
B
0 100 500 1000 2000 U/ml
IDO mRNA
18 S

Figure 2.3

C


Expression of IDO mRNA/18S
120
100
80
60
40
20
0
*
*
*
0
100
500
1000
2000
IFN-γ concentration (U/ml)

D

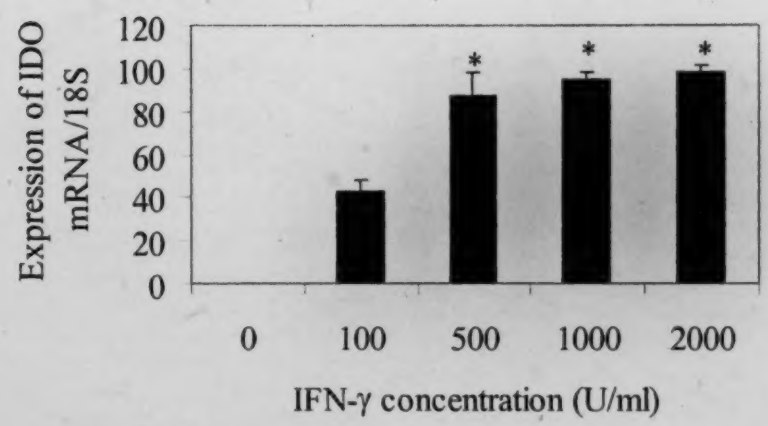

Expression of IDO mRNA/18S
120
100
80
60
40
20
0
*
*
*
0
100
500
1000
2000
IFN-γ concentration (U/ml)

Figure 2.3

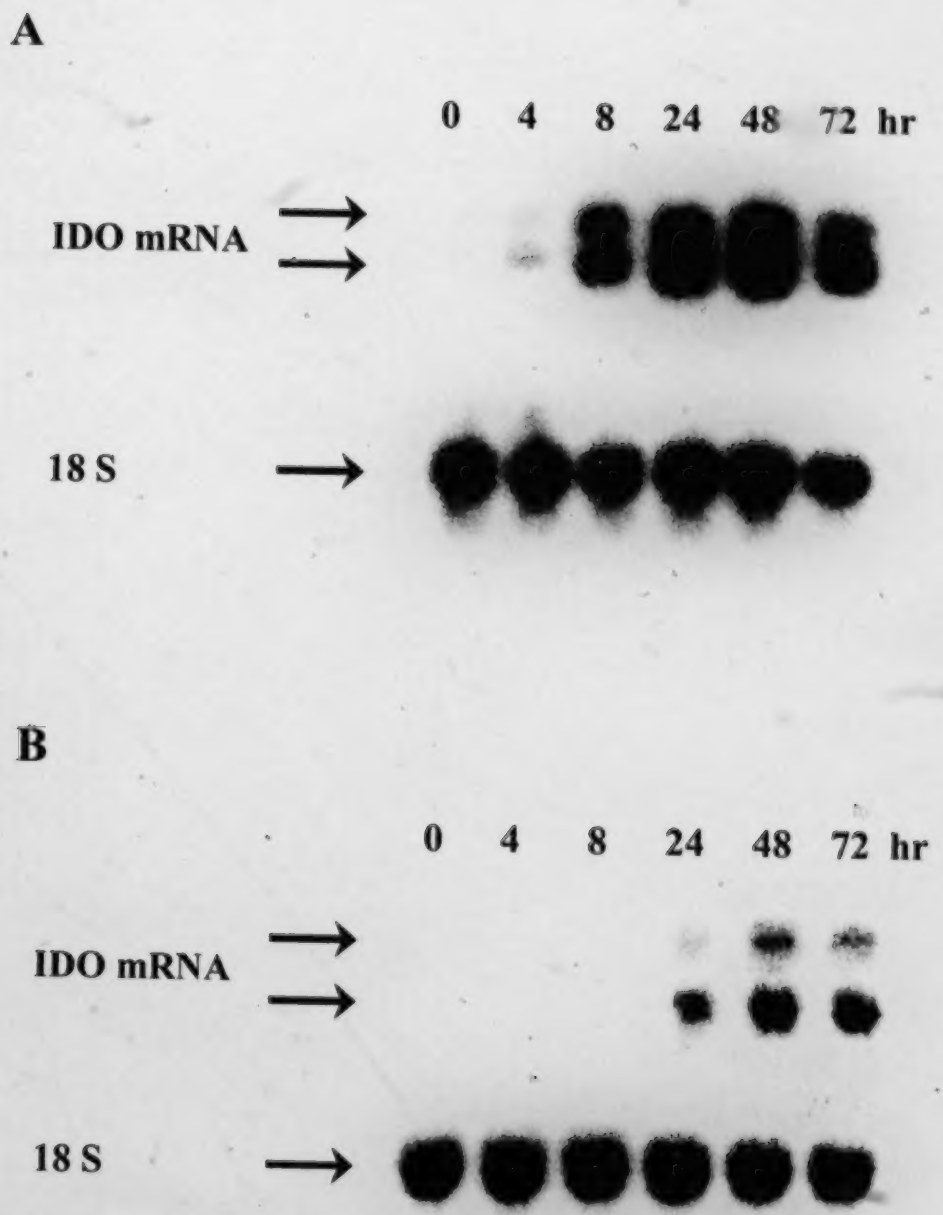
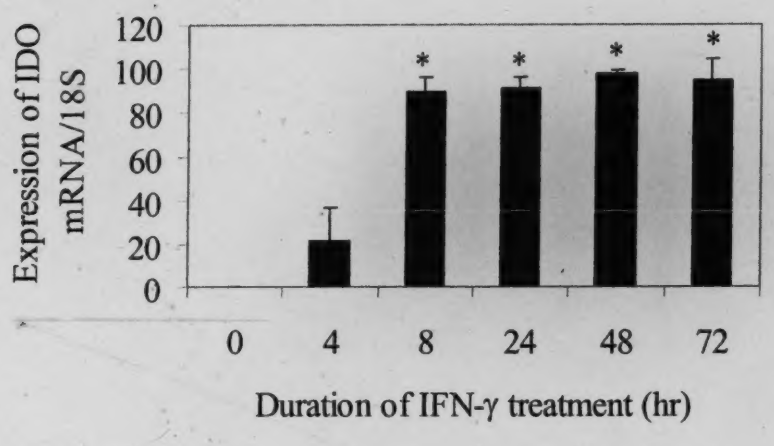


Figure 3.3

C


Expression of IDO mRNA/18S
120
100
80
60
40
20
0
*
*
*
*
0
4
8
24
48
72
Duration of IFN-γ treatment (hr)

D

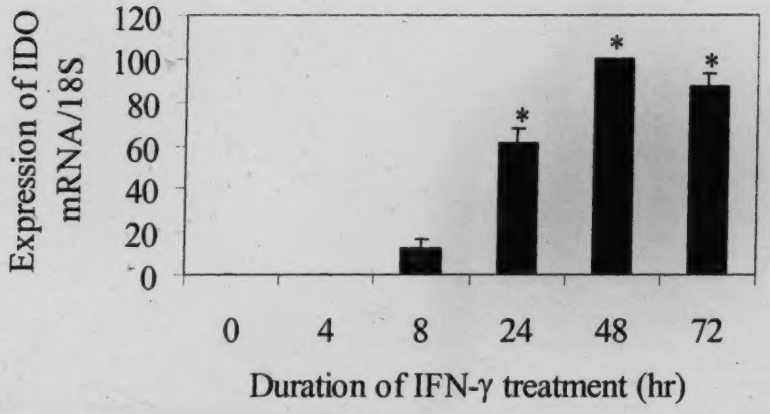
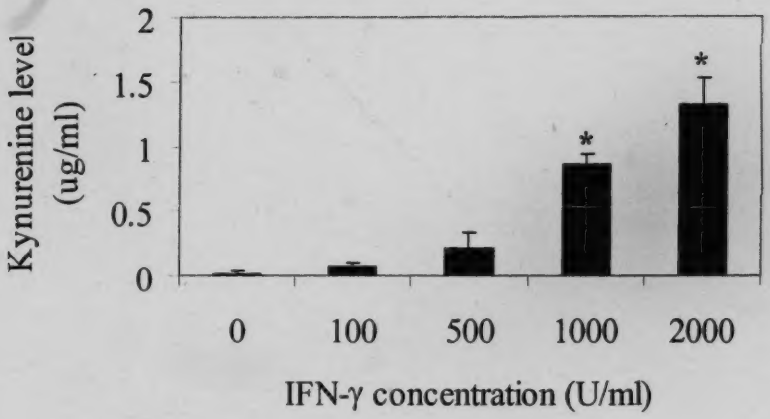

Expression of IDO mRNA/18S
120
100
80
60
40
20
0
*
*
*
0
4
8
24
48
72
Duration of IFN-γ treatment (hr)

Figure 3.3

A


Kynurenine level (ug/ml)
2
1.5
1
0.5
0
*
*
0
100
500
1000
2000
IFN-γ concentration (U/ml)

B

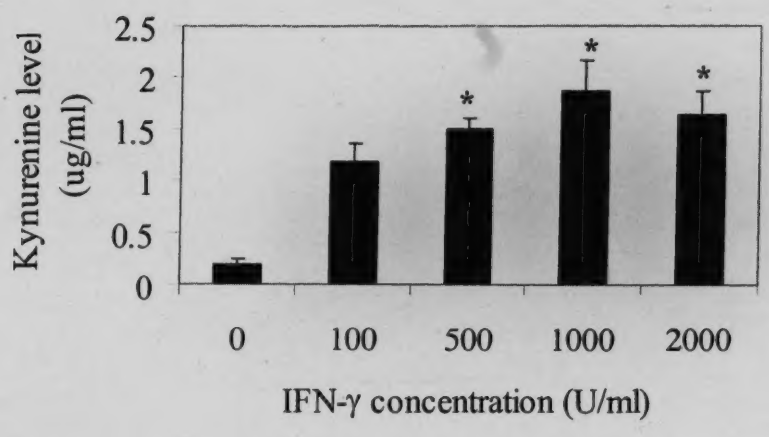
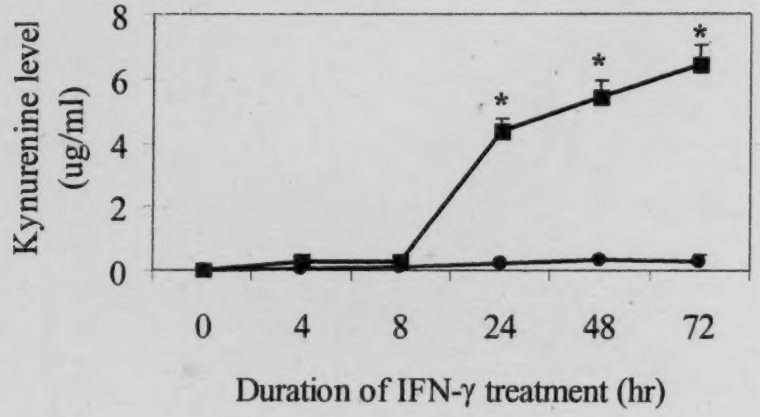

Kynurenine level (ug/ml)
2.5
2
1.5
1
0.5
0
*
*
*
0
100
500
1000
2000
IFN-γ concentration (U/ml)

Figure 4.3

C


Kynurenine level (ug/ml)
8
6
4
2
0
*
*
*
0
4
8
24
48
72
Duration of IFN-γ treatment (hr)

D

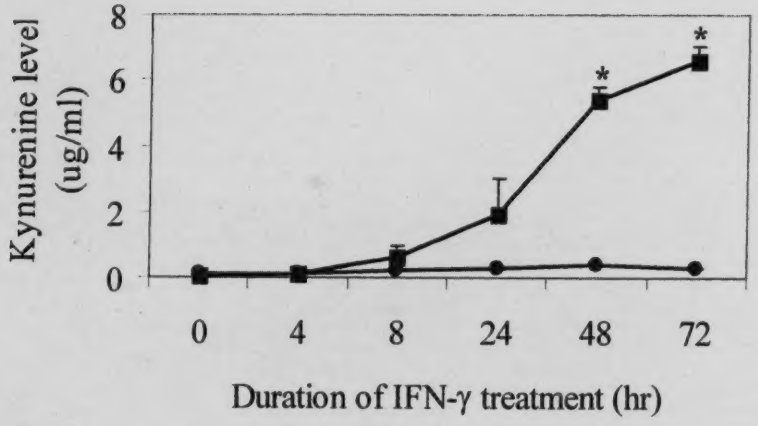

Kynurenine level (ug/ml)
8
6
4
2
0
*
*
0
4
8
24
48
72
Duration of IFN-γ treatment (hr)

Figure 4.3

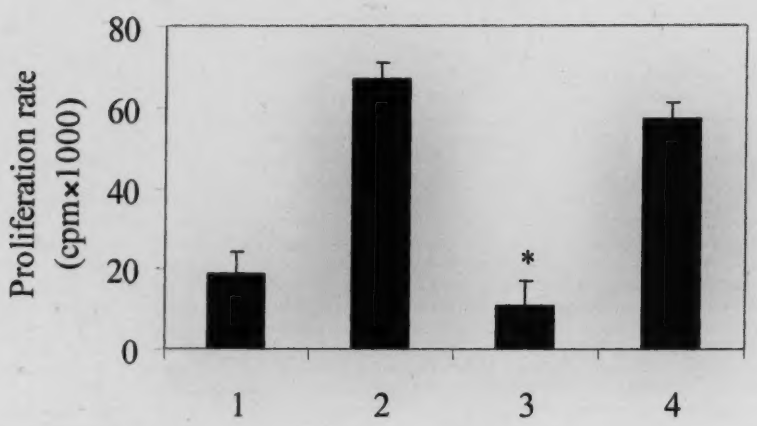


Figure 5.3

CHAPTER FOUR:

IMMUNE CELL PROLIFERATION IS SUPPRESSED BY THE INTERFERON-γ-INDUCED INDOLEAMINE 2,3-DIOXYGENASE EXPRESSION OF FIBROBLASTS POPULATED IN COLLAGEN GEL (FPCG) *

* A version of this chapter has been published in the Journal of Cellular Biochemistry 90(1): 206-217, 2003

ABSTRACT

Indoleamine 2,3-dioxygenase (IDO), a tryptophan-catabolizing enzyme, is an intracellular enzyme possessing various immunosuppressive properties. Here, we report the possible use of this enzyme to suppress proliferation of immune cells cocultured with IDO-expressing fibroblasts of an allogeneic skin substitute. Fetal foreskin fibroblasts embedded within bovine collagen were treated with cytokine interferon-γ (IFN-γ) to induce expression of IDO mRNA and protein. Expression of IDO mRNA was evaluated by Northern analysis. IDO enzyme activity was evaluated by measurement of kynurenine and tryptophan levels in the IFN-γ-treated fibroblasts. The results of Northern analysis showed a dose-dependent increase in expression of IDO mRNA in response to various concentrations of IFN-γ used. The levels of kynurenine and tryptophan, as the measurement for the bioactivity of IDO, were significantly different in the IFN-γ treated fibroblasts, compared to those of controls ($p<0.001$). In a lasting effect experiment, the expression of IDO mRNA was gradually reduced to an undetectable level within 32 hr of IFN-γ removal. The results of western blot analysis, however, revealed a significantly longer (192 hr) lasting effect of IFN-γ on IDO protein level, relative to that of mRNA expression. To demonstrate immunosuppressive effects of IDO on proliferation of immune cells, IDO-expressing fibroblasts were cocultured with peripheral blood mononuclear cells (PBMC) for a period of 5 days. The results of ^{3}H-thymidine incorporation showed a significant reduction in proliferation of PBMC when cocultured with IDO-expressing fibroblasts, compared to those cocultured with non IDO-expressing fibroblasts ($p<0.001$). Furthermore, addition of IDO-inhibitor (1-methyl-D-tryptophan) reversed the suppressive effects of IDO on PBMC proliferation in a dose-dependent manner. To test the viability of

immune cells cocultured with IDO-expressing fibroblasts, FACS analysis of the PI-stained PBMC was conducted and no significant difference was found between these cells and the controls. In another set of experiments, we showed that migration rate and subsequent proliferation of IDO-expressing fibroblasts are also the same as those of control cells. In conclusion, IDO-expressing allogeneic fibroblasts embedded within collagen gel suppress the proliferation of allogeneic immune cells while still remaining viable in this IDO-induced tryptophan-deficient culture environment.

INTRODUCTION

Extensive skin loss from a variety of conditions such as severe thermal injury is associated with significant functional morbidity and mortality (Cairns et al., 1993). In recent years, however, the overall mortality rate has improved for patients suffering from burns due in part to the significant biotechnological advancements in skin replacement for wound closure (Cairns et al., 1993). *In vitro* cultivation of keratinocytes with the support of a feeder layer of lethally irradiated 3T3-cells was initially introduced by Rheinwald and Green (1975). These investigators were later able to grow keratinocytes to confluency, which was suitable for grafting (Gallico et al., 1984). Although sheets of autologous keratinocytes are currently used in some burn centers to treat patients with large thermal injury (Morthenn et al., 1982; Thivolet et al., 1986), this has not been a routine procedure for several reasons: 1) sheets of keratinocytes prepared from layers of cultured keratinocytes without matrix are very fragile and difficult to cultivate, 2) the rate of graft-take is relatively low (50-60%), 3) patients with large injuries do not have enough uninjured skin to be used for cell culture, 4) leaving the burn wound open for 3-6 weeks until cells become available increases the risk of infections, heat or fluid loss, and finally, 5) generating another wound increases the risk of developing hypertrophic scarring. Considering that all these are pervasive medical problems with far-reaching clinical and economic implications, utilizing an allogeneic and readily available skin substitute would be beneficial. Therefore, we have designed and recently conducted a series of experiments to examine the benefit of using allogeneic fibroblasts expressing indoleamine 2,3-dioxygenase as a local immunosuppressive factor.

Indoleamine 2,3-dioxygenase (IDO) is an intracellular enzyme that converts tryptophan, the least available essential amino acid, into N-formylkynurenine. N-formylkynurenine is then converted to L-kynurenine by the enzyme formamidase (Takikawa et al., 1986). IDO-induced tryptophan depletion has been illustrated to play important roles in physiological conditions such as the suppression of T-cell responses *in vivo* and *in vitro* (Mellor et al., 2002). It also prevents maternal T-cell mediated rejection of allogeneic fetuses (Mellor et al., 2001; Munn et al., 1998) and prolongs graft survival of IDO-expressing pancreatic islet cells (Alexander et al., 2002). Expression of IDO by human trophoblasts of the placenta in the contact zone between the maternal and fetal immune cells protects the fetus from the maternal immune cell rejection (Kamimura et al., 1991). The immunosuppressive role of IDO has been demonstrated by showing suppression of T-cell proliferation in the vicinity of IDO-expressing macrophages. It has been suggested that local tryptophan deprivation may play a key role in suppression of immune cell proliferation (Munn et al., 1999). Induction of IDO is specific to IFN-γ, but not to interferon α or β (Takikawa et al., 1988) and this cytokine, therefore, has been used to induce IDO expression in dermal fibroblasts.

Interferon-gamma (IFN-γ), a pleotropic cytokine secreted by natural killer (NK) and activated T cells, is an important component of our immune system (Billiau and Dijkamans, 1990; Boehm et al., 1997). IFN-γ exerts its effects by binding to specific receptors on human fibroblasts (Anderson et al., 1982), which activate the Jak-STAT signal transduction pathway (Sadir et al., 2000). Activated STAT1 is then translocated to the nucleus and binds to response elements at the promoter region of the target gene (Heim, 1999). The IFN-γ-receptor complex is finally internalized and subsequently

degraded (Anderson et al., 1982). IDO expression is shown to be halted in IFN-γ resistant mutant cell lines (Feng and Taylor, 1989), which further supports the crucial role of this cytokine in stimulation of IDO expression and production. The IFN-γ-induced IDO expression has been shown previously to possess anti-parasitic (Dai et al., 1994), anti-viral (Cheney et al., 2002), anti-tumour (Takikawa et al., 1990; Burke et al., 1995) and anti-proliferative (Maza and Peterson, 1988) activity. The anti-proliferative activity of IFN-γ depends on the tryptophan concentration and can be reversed by the addition of tryptophan in a dose- and time-dependent manner (Leung et al., 1992; Ozaki et al., 1988).

Considering the role of IDO in protecting maternal T-cell mediated rejection of allogeneic fetuses (Mellor et al., 2001; Munn et al. 1998) and the success of IDO in prolonging survival of pancreatic islet cells (Alexander et al., 2002), we hypothesize that IDO could function as a local immunosuppressive factor to protect allogeneic skin substitutes. In this study, therefore, we evaluate the proliferation of human peripheral blood mononuclear cells cocultured with IFN-γ-induced IDO-expressing dermal fibroblasts. Our findings reveal that IFN-γ induces the expression of IDO mRNA and its enzyme activity. This generates a tryptophan-deficient microenvironment in which human peripheral blood mononuclear cells (PBMC) are unable to proliferate. In contrast to immune cells, however, IFN-γ-induced IDO-expressing dermal fibroblasts are able to migrate out of the collagen gel and proliferate at the same rate as the IFN-γ-untreated control cells.

METHODS AND MATERIALS

Fibroblast Cell Culture:

Cultures of human foreskin fibroblasts were established as described previously by Ghahary et al. (1997). In brief, punch biopsy samples were prepared from human foreskin. The tissue was collected in DMEM with 10% FBS (GIBCO, Grand Island, NY), minced into small pieces of less than 0.5 mm in any dimension, washed with sterile medium six times, and distributed into 60×15 mm Petri culture dishes (Corning Inc., Corning, NY), four pieces per dish. A sterile glass cover-slip was attached to the dish with a drop of sterile silicone grease to immobilize the tissue fragment. DMEM + Ab (penicillin G sodium 100 U/ml, streptomycin sulfate 100 μg/ml, and amphotericin B 0.25 μg/ml) (3ml) with 10% FBS was added to each dish and incubated at 37°C in a water-jacketed humidified incubator in an atmosphere of 5% CO_2. The medium was replaced twice weekly. After 4 weeks of incubation, cells were released from dishes by brief (5 min) treatment with 0.1% trypsin (Life Technologies Inc., Gaithersburg, MD) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) in PBS (pH 7.4) and transferred to 75 cm^2 culture flasks (Corning Inc., Corning, NY). Thereafter, once visual confluence was reached, the cells were subcultured 1:6 by trypsinization. Fibroblasts from passages 3 to 7 were used for this study.

Fibroblasts Populated in Collagen Gel:

Utilizing a modification procedure of Bell et al. (1979), fibroblasts populated in collagen gel (FPCG) were prepared using bovine type I procollagen extracted in our laboratory by a procedure described by Volpin and Veis (1971). Experiments were performed using six-well plates (ICN Biomedicals Inc., Aurora, OH) with 35 mm

diameter. Each well contained 350 µl 3×DMEM + Ab, 26 µl 0.4 N NaOH, 440 µl cell suspension (2×10^6 cells/ml) in 1×DMEM + 10% FBS, 125 µl FBS (for a final concentration of 10%) and 870 µl of acid-extracted fetal bovine type I collagen (2.14 mg/ml). Each treatment group was prepared in triplicates and was immediately transferred to a humidified incubator at 37°C in an atmosphere of 5% CO_2.

IFN-γ Treatment:

Fibroblasts populated in collagen gel (FPCG) were placed in the incubator for 3 hr in order for the collagen gel lattices to be formed. After this period, collagen gels were released from the plates using a surgical blade (Fisher Scientific, Edmonton, AB), and fibroblasts were treated with 2000 U of human recombinant IFN-γ (Sigma, Saint Louis, MO) for a period of 48 hr. After this period, in order to find the duration of the effect of IDO expression after IFN-γ removal, conditioned medium was replaced with a fresh medium containing no IFN-γ. Cells were harvested at different time-points post IFN-γ removal. Conditioned medium was also collected and used to determine the levels of kynurénine and tryptophan.

Northern Blot Analysis:

Fibroblasts populated in collagen gel (FPCG) were harvested and the collagen lattice was digested with bacterial collagenase (Sigma, St. Louis, MO) in PBS (4mg/10ml) at 37°C for 30 min. Isolated cells were pelleted by centrifugation at 1100 rpm for 10 min. Pellets were then lysed with 500 µl of 4 M guanidium isothiocyanate (GITC) solution and total RNA from each group was isolated by the guanidium

isothiocyanate/CsCl procedure of Chirgwin et al. (1979) using phenol:chloroform (1:1), followed by chloroform:isoamyl alcohol (49:1). Total RNA from each individual fibroblast culture was then separated by electrophoresis (10 μg per lane) on a 1% agarose gel containing 2.2 M formaldehyde and was blotted onto a nitrocellulose paper. To control the loading, quantities of 18S ribosomal RNA were compared visually by ethidium bromide fluorescence. The blots were baked for 2 hr at 80°C under vacuum and prehybridized for 4 hr at 45°C in a prehybridization solution. Hybridization was performed at 45°C in the same solution, using IDO, 18S or type I procollagen cDNA. The cDNA probes were labeled with P-α^{32}-dCTP by nick translation. Filters were washed initially at room temperature with 2×sodium citrate/sodium chloride buffers and 0.1% sodium dodecylsulfate for 1 hr and then rewashed for 20 min at 65°C in 0.1×sodium citrate/sodium chloride buffer and 0.1% sodium dodecylsulfate. Autoradiography was performed by exposing Kodak X-Omat film to nitrocellulose filters at 80°C in the presence of an intensifying screen. Quantitative analysis of autoradiographs was accomplished by densitometry. The cDNA probe for 18S was obtained from the American Type Culture Collection (Rockville, MD). The other cDNA probes were gifts: IDO (Dr. J.M. Carlin, Department of Microbiology, Miami University, Oxford, OH, USA) and type I procollagen (Pro α_1 (I)) (Drs. G. Tromp, H. Kuivaneimi, and L. Ala-Kokko, Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Medicine, Philadelphia, PA).

Determination of Total Kynurenine and Tryptophan in the Conditioned Medium:

The biological activity of IDO was evaluated by measuring the level of tryptophan or its degradation product, kynurenine, present in the conditioned medium derived from IFN-γ treated fibroblasts. Conditioned medium related to the same number of cells was first deproteinized by adding 100 μL of acetone to 50 μL of the cell culture medium. The sample was vortexed, cooled on ice and centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant of each sample received 10 μl of 0.25 M $HClO_4$ and internal standard (4-amino-hippuric acid) solution, and the mixture was incubated at 25°C for 25 min. The mixture was then evaporated to dryness under vacuum. 100 μL of 14% BF_3-propanol was added to the residue in a screw-capped glass tube and was heated at 90 °C for 2 hr. The solution was again evaporated to dryness under vacuum. The residue was dissolved in 40 μL of 20% MeOH in water and was analyzed by liquid chromatography with electrospray mass spectrometry (LC/EMS) using a Hewlett Packard series 1100 mass selective detector controlled by 1100-MED Chem Station. The mass spectrometer was operated in the positive ion mode. Tryptophan and kynurenine quantitative analyses were performed in the selected ion-monitoring mode.

Western Blot Analysis:

To measure the persistence of IDO expression in dermal fibroblasts following the removal of interferon-γ, western blot analysis was performed on the harvested fibroblasts every 48 hr post interferon-γ removal for a total period of 240 hr. Briefly, cell extracts were prepared by adding 2×10^6 fibroblasts to the lysate buffer (50 mM Tris-HCl [pH=7.40], 150 mM NaCl, 1mM EDTA (Sigma, St. Louis, MO), 1mM EGTA (Sigma-Aldrich Canada, Ltd.), 0.025% NaN_3, 1% TritonX-100, 0.5% Igepal CA-630 (Sigma, St.

Louis, MO), and protease inhibitor cocktail (Sigma, St. Louis, MO)). Extracts were centrifuged at 13,000 rpm for 10 min at 4°C, and 50 μg (BSA protein assay [Pierce]) of the total protein was run on a 10% SDS-PAGE. Proteins were then transferred to a PVDF membrane (Millipore Corp., Bedford, MA) by Mini Trans-Blot Cell (Bio-Rad Laboratories Ltd., Mississauga, ON). Blots were incubated with either IDO (raised in our laboratory) or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) monoclonal primary antibodies. Horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma, St. Louis, MO) was used and visualized using ECL+Plus™ western blotting detection system (Amersham Biosciences).

Peripheral Blood Mononuclear Cell (PBMC) Isolation and Mixed Lymphocyte Reaction:

Samples of 25 to 30 ml of human blood were drawn into heparinized tubes from normal individuals. Total PBMC was isolated by density gradient sedimentation on Histopaque 1077 (Sigma, St. Louis, MO) using the manufacturer's protocol. Briefly, whole blood was layered onto an equal volume of Histopaque and centrifuged at 2000 rpm for 20 min at room temperature. Peripheral mononuclear blood cells were isolated and added to RPMI 1640 + 10% FBS (GIBCO, Grand Island, NY). They were then pelleted by centrifugation at 2000 rpm for 10 min and washed twice more in RPMI 1640. For the mixed lymphocyte reaction experiment, fibroblasts were treated with 2000 U of IFN-γ for a period of 48 hr, after which IFN-γ was removed. The cells were then washed three times with PBS and the conditioned medium was replaced with fresh medium (with no IFN-γ). Subsequently, 5×10^4 fibroblasts were plated into twelve-well plates (Costar,

Cambridge, MA), irradiated with 2000 units of gamma rays and cocultured with 2×10^5 of PBMC in RPMI 1640 + 10% FBS for a period of 5 d. To show the specificity of IDO action on proliferation of PBMC, the IDO inhibitor 1-methyl-D-tryptophan (Aldrich Chem. Co., Milwaukee, WI) was added to coculture samples in different concentrations of 50, 100, 200, 400 and 800 μM.

^{3}H-thymidine Incorporation Assay:

To measure the rate of peripheral blood mononuclear cell (PBMC) proliferation in response to exposure to allogeneic fibroblasts, ^{3}H-thymidine proliferation assay was performed on the PBMC cocultured with fibroblasts. In brief, ^{3}H-thymidine (PerkinElmer Life Sciences Inc., Boston, MA) was added to conditioned medium of each sample for a final concentration of 2 μCi/ml and was incubated for 16 hr. After this period, PBMC were harvested, washed three times with PBS, dissolved in GITC, and added to the scintillation fluid (Amersham Canada Ltd, Oakville, ON). Radioactive counting was performed using the scintillation counter (Beckman) and measured in counts per min (cpm). PBMC proliferation rate was then calculated by dividing the obtained radioactive counts by the highest radioactive count for each set of experiments.

Fluorescence-Activated Cell Sorting (FACS) Analysis:

To determine whether IDO expression in fibroblasts has any effects on the cocultured PBMC viability, propydium iodide (PI) staining followed by flow cytometry was performed to measure the ratio of dead (stained) to live cells. Harvested blood cells were stained with 10 μg/ml of propydium iodide (PI) (Sigma, St. Louis, MO) for 10 min

and washed twice with PBS. Flow cytometry was used to quantify the relative number of cells stained for the PI.

Fibroblast Gel Migration and Proliferation Assay:

To investigate the effects of IDO expression on the rate of fibroblast migration from the skin substitute and on their subsequent proliferation, fibroblast migration and proliferation assays were performed. Briefly, 6 mm punch biopsies were taken from the IFN-γ treated and untreated FPCG. Punch biopsies were placed in triplicates onto six-well plates. The number of fibroblasts migrating out of the gel was measured every 2 d for a period of 8 d by removing the punch biopsies and counting the number of attached cells using a hemocytometer.

Statistical Analysis:

Student's unpaired two-tailed t-test was used to evaluate the significance of differences in the levels of tryptophan and kynurenine, the rate of fibroblast migration and proliferation, se well as PBMC proliferation and densitometry results of western and Northern analyses between IFN-γ treated and untreated fibroblasts.

RESULTS

IFN-γ induces IDO expression in fibroblasts in a dose-dependent manner:

Northern blot analysis of the total RNA extracted from IFN-γ treated FPCG revealed that IDO expression is markedly induced at the IFN-γ concentration of 1000 U/ml and this expression is further increased with increasing concentrations of IFN-γ.

Fibroblasts normally do not express detectable levels of IDO mRNA; however, upon treatment with IFN-γ, IDO expression was markedly induced in the skin fibroblasts (Fig. 1.4A). Combined densitometry results from three separate experiments revealed that IFN-γ reduces type I procollagen mRNA/18S and increases IDO mRNA/18S ratios in a dose-dependent fashion in the FPCG (Fig. 1.4B).

Different strains of FPCG were also either untreated (control) or treated with 2000 U of IFN-γ for a period of 48 hr. Northern blot analysis of the total RNA extracted from four different strains of human fibroblasts revealed that IDO expression is inducible at the mRNA level in all fibroblast strains upon IFN-γ treatment (Fig. 2.4A). Combined densitometry results from four separate experiments showed that IFN-γ markedly increases the expression of IDO mRNA/18S (Fig. 2.4B) and significantly decreases the expression of type I procollagen mRNA/18S ratios ($p<0.001$, $n=4$) (Fig. 2.4C).

IDO expression reduces in fibroblasts upon IFN-γ removal:

Fibroblasts populated in collagen gel, treated with 2000 U of IFN-γ for 48 hr, showed a high level of IDO mRNA expression, which was gradually reduced upon IFN-γ removal up to the 32 hr time-point examined. Representative Northern blot analysis of the total RNA extracted from fibroblasts at 0, 8, 16, 24 and 32 hr post IFN-γ removal is depicted (Fig. 3.4A). Densitometry results from three separate experiments revealed a reduction in IDO mRNA expression in fibroblasts after the interferon-γ removal (Fig. 3.4B). Western blot analysis of IDO in fibroblasts treated with 2000 U of IFN-γ illustrated that collagen embedded fibroblasts express IDO even at 192 hr post interferon-γ removal (Fig. 3.4C). Combined densitometry results from three separate experiments

also showed that IDO protein level was reduced in the collagen embedded fibroblasts after IFN-γ removal (Fig. 3.4D).

IFN-γ-induced IDO expression increases kynurenine and decreases tryptophan levels:

To measure the enzyme activity of IDO in fibroblasts populated in collagen gel, tryptophan and kynurenine levels were measured in the collected conditioned media after treatment with different doses of IFN-γ for 48 hr. Fibroblasts treated with 500, 1000, 2000 and 4000U of IFN-γ had higher levels of kynurenine (10.01 ± 0.94, 10.72 ± 1.46, 10.68 ± 1.43 and 11.05 ± 0.25 μg/ml, respectively) compared to the untreated (control) fibroblasts (0.93 ± 0.58 μg/ml) (* $p < 0.001$, n=3) (Fig. 4.4A). Data from four separate experiments showed that fibroblast treatment with 2000 U of IFN-γ for 48 hr decreases tryptophan levels (4.96 ± 1.74 vs 17.56 ± 1.23 μg/ml) (* $p<0.001$, n=4) (Fig. 4.4B) and increases kynurenine levels (14.68 ± 2.14 vs 1.29 ± 0.16 μg/ml) in fibroblast conditioned medium (* $p<0.001$, n=4) (Fig. 4.4C). The relative production level of kynurenine in fibroblasts remained high at 32 hr post IFN-γ removal (94 ± 6%), which was significantly higher than that in control samples (9 ± 4%) (* $p<0.001$, n=3) (Fig. 4.4D).

IDO-expressing fibroblasts suppress PBMC proliferation in a coculture system:

The ^{3}H-thymidine incorporation assay revealed that PBMC proliferation is significantly reduced when cocultured with IDO-expressing fibroblasts. Furthermore, addition of IDO inhibitor (1-methyl-D- tryptophan) reversed the inhibitory effects of IDO on lymphocyte proliferation in a dose-dependent manner, up to a point where PBMC proliferation rate

was not significantly different between IDO-expressing and non IDO-expressing (control) fibroblasts at the concentration of 800 μM 1-methyl-D-tryptophan (*, ** $p < 0.05$ and <0.001, respectively, n=3) (Fig. 5.4A). The result of FACS analysis on the viability of PBMC cultured alone, or in the vicinity of either IDO-expressing or non-expressing fibroblasts showed that PBMC viability is not significantly different amongst these three cell populations examined (Fig. 5.4B).

IDO-expressing fibroblasts can migrate and proliferate despite the tryptophan depletion:

The results of fibroblast migration assay revealed that IDO-expressing fibroblasts are able to migrate out of the collagen gel (Fig. 6.4A). Fibroblast proliferation assays of IDO-expressing and non-expressing fibroblasts also showed that the rate of fibroblast proliferation was not significantly different between IDO-expressing and control cells (Fig. 6.4B).

DISCUSSION

The ultimate goal of any tissue engineering-related study is to reduce the clinical complications of both non-healing and over-healing wounds. As such, there is a need to develop a non-rejectable and readily available skin substitute. This skin substitute must have both epidermal and dermal layers to have an acceptable biological function. It would function as early wound coverage to prevent or reduce heat and fluid loss, and to prevent wound infection. The skin substitute should also permit the release of dermal-epidermal wound healing modulating factors locally to facilitate granulation tissue

formation and re-epithelialization. As well, this would improve the closure of non-healing wounds, such as those seen in the diabetic patients.

Though desirable, it is unlikely that autologous engraftments can be obtained for patients who suffer from extensive skin loss from large and severe thermal injuries, due to limited amounts of uninjured tissue remaining. The same is true for diabetic, elderly and immuno-compromised patients who suffer from non-healing complications. Furthermore, preparation of an autologous skin substitute is time-consuming, thereby reducing the benefit for patients whose survival depends on an early application of wound coverage. As an alternative, the use of non-living extracellular matrix substitutes could be examined, but these are not capable of releasing wound healing promoting factors. Therefore, exploring an allogeneic, non-rejectable, and readily available skin substitute may provide a better means of improving wound healing. As rejection is a major obstacle in any type of grafting, the current study seeks a novel approach through which the local induction of immunosuppressive factors, such as indoleamine 2,3-dioxygenase (IDO), generates a tryptophan-deficient microenvironment. Such an environment could prevent infiltrated immune cells from proliferating and destroying the grafted skin substitutes.

We have established here that dermal fibroblasts grown in a three-dimensional collagen gel are highly responsive to IFN-γ-induced IDO mRNA expression. As IDO catalyzes the conversion of an essential amino acid tryptophan to kynurenine (Higuchi et al., 1967; Hayaishi, 1996; Munn et al., 1998), and as the presence of tryptophan is required for protein synthesis, stimulated immune cells would not be able to proliferate in an environment with such a low level of tryptophan.

Previous studies have shown that IDO expression in different cell types can also inhibit immune cell proliferation (Hwu P et al., 2000; Logan GJ et al., 2002). However, studying the immunosuppressive properties of IDO in dermal fibroblasts situated in a three-dimensional system is more similar to an *in vivo* system and should be investigated. The results of the mixed lymphocyte reaction assay showed that IFN-γ-induced IDO in dermal fibroblasts suppresses the proliferation of stimulated PBMC. This suppression is completely restored by the addition of an IDO inhibitor (1-methyl-D-tryptophan) at 800 μM concentration. The results of FACS analysis indicated that IFN-γ-induced IDO expression does not reduce the viability of PBMC.

IDO expression generates a tryptophan-depleted environment in which immune cells are unable to proliferate without compromising skin cell viability. Although our findings suggest that immune cells cocultured with IDO-expressing fibroblasts are unable to proliferate, a key concern is whether IFN-γ-induced IDO-expressing cells themselves are able to proliferate in the same environment. In IDO adenoviral infected cells, we have recently demonstrated that there is a marked difference in the responsiveness to tryptophan-depletion amongst different immune cell types (unpublished data). In fact, $CD4^+$ cells, which play a major role in graft rejection, are very sensitive to low-tryptophan levels as more than 82 % of them died in low-tryptophan culture conditions. However, THP-1 cells (monocytes) are significantly less sensitive in the same environment. In the same study, we also showed that IDO adenoviral-infected 293 cells with high levels of IDO expression attach to the bottom of culture dishes and survive better than immune cells, as evaluated by FACS analysis of PI-stained cells. These findings indicate that cells with different proliferation abilities may respond differently to

tryptophan-deficient environments. In fact, as indicated by fibroblast migration from collagen gels and their subsequent proliferation, primary cultured fibroblasts appear to be less sensitive to an IDO-induced, low-tryptophan environment. The pattern of cell migration and proliferation of FPCG was comparable between IFN-γ-treated and -untreated fibroblasts. A better survival of IDO-expressing cells relative to that of highly proliferative immune cells is not surprising because IDO-expressing cells, such as the trophoblasts of the placenta, survive better than infiltrated immune cells during pregnancy. Munn et al. (1999) recently reported that antigen-presenting cells can regulate T-cell activation through tryptophan catabolism. They speculated that the expression of IDO by certain antigen-presenting cells *in vivo* allowed them to suppress unwanted T-cell responses. These investigators demonstrated that the expression of IDO during murine pregnancy is required to prevent the rejection of allogeneic fetuses by maternal T-cells.

The result of the lasting effect of IFN-γ on the expression of IDO mRNA in dermal fibroblasts revealed that IFN-γ-induced IDO expression in treated cells is transient. The level of IDO mRNA expression in treated cells returned to undetectable levels within 2 days after IFN-γ removal. The duration of effect of IFN-γ treatment on the presence of IDO protein is significantly longer (more than 6 days) than that of IDO mRNA expression. This finding suggests that IDO protein is stable and can be stored within cells for a longer period of time.

To further prolong IFN-γ-induced IDO expression, we are currently examining the use of IFN-γ protein-conjugated polymers, since a slow release of IFN-γ may be necessary to sustain level of IDO expression that is adequate for the protection of grafts.

The slow release of IFN-γ may also be beneficial for those patients who suffer from severe thermal injury, since we have recently found that the level of IFN-γ in patients with severe burn injury is significantly lower than in normal controls (data not shown). As well, the lower level of IFN-γ, an anti-fibrotic factor, may contribute to the development of hypertrophic scarring that is frequently seen upon severe thermal injury. In fact, the use of IFN-γ as a therapeutic agent to treat dermal fibrotic conditions has been suggested due to previous evidence that IFN-γ reduces both fibroblast proliferation and the synthesis of type I and III collagen (Harrop et al., 1995). Furthermore, according to Halloran et al. (2001), engraftment of the kidney in IFN-γ knockout mice is accompanied by an increase in vascular injury, congestion of pretubular capillaries, and massive necrosis of parenchymal cells. Thus, the use of IFN-γ may be beneficial in preventing thrombosis, congestion and necrosis of grafted tissues, and more likely, less scar formation.

In conclusion, we have demonstrated that IFN-γ induces the expression of IDO mRNA and protein in dermal fibroblasts grown in a three-dimensional collagen environment. The resultant tryptophan-deficiency creates an environment in which stimulated immune cells are unable to proliferate. Therefore, this approach may be useful for the use of local immunosuppressive factors such as IDO in the development of non-rejectable skin substitutes.

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FIGURE LEGENDS

Figure 1.4. IFN-γ-induced expression of IDO mRNA in fibroblasts populated within collagen gel (FPCG) is dose-dependent. Fibroblasts were treated with either 0 (control), 500, 1000, 2000 or 4000 U of IFN-γ for 48 hr. The total RNA was extracted and Northern blot analysis was performed to determine the expression of mRNA for IDO and type I procollagen. The same blot was then re-hybridized with cDNA specific for 18S ribosomal RNA, which was used as a RNA loading control. **Panel A**, depicts the pattern of IDO and type I procollagen mRNA expression in fibroblasts treated with various concentrations of IFN-γ. **Panel B**, shows the densitometry data on the ratio of either IDO mRNA/18S (squares) or type I procollagen mRNA/18S (triangles) as a function of various concentrations of IFN–γ.

Figure 2.4. IDO mRNA expression is inducible in different fibroblast strains upon IFN-γ treatment. Four different strains of human fibroblasts were either untreated (control) or treated with 2000 U of IFN-γ for 48 hr and Northern analysis was then performed to evaluate the expression of IDO and type I procollagen mRNA using 18S ribosomal RNA as a loading control (**Panel A**). The ratio of either IDO mRNA/18S rRNA or type I procollagen mRNA/18S rRNA of each autoradiogram was then determined and the mean +/- SD was calculated and presented in **Panels B** and **C**, respectively. The p-value (* $p < 0.001$) for collagen mRNA expression between treated and untreated fibroblasts is considered to be extremely significant. Statistical evaluation on IDO mRNA expression, however, was not performed because the level of IDO mRNA expression was not detectable in all four different strains of untreated fibroblasts.

Figure 3.4. IDO expression in fibroblasts populated in collagen gel is reduced after IFN-γ removal. FPCG were treated with 2000 U of IFN-γ for 48 hr. The conditioned medium was then replaced with a fresh medium with no IFN-γ. Cells were harvested at 0, 8, 16, 24, and 32 hr post IFN-γ removal and total RNA was extracted and subjected to Northern analysis. **Panel A** represents the pattern of IDO mRNA expression and 18S ribosomal RNA in fibroblasts untreated (C), or treated with IFN-γ for 48 hr and harvested at the indicated time-points post IFN-γ removal. Densitometry of autoradiograms related to three separate experiments was used to determine the ratio of IDO mRNA expression/18S ribosomal RNA. Data is expressed as the mean +/- SD for each time point (**Panel B**).

Panel C, represents the pattern of IDO protein detected by western blot analysis of collagen embedded fibroblasts harvested from either IFN-γ untreated (C), or treated for 48 hr and harvested at the indicated time-points post IFN-γ removal. The presence of β-actin in the same samples was also evaluated as a protein loading control. The intensity of each IDO protein band of western blot was then determined by densitometry and data is expressed as the ratio of IDO /β-actin (Panel D). Data is shown the mean +/- SD for three separate experiments.

Figure 4.4. Kynurenine and tryptophan levels measured in the conditioned medium of FPCG. A decreased level of tryptophan in the conditioned medium collected from IFN-γ-induced IDO-expressing fibroblasts was inverse to the level of kynurenine. **Panel A**, fibroblasts populated in collagen gel were treated with either 0 (control), 500, 1000,

2000 or 4000 U of IFN-γ and the conditioned medium was collected and evaluated for the kynurenine content. For statistical analysis, in a similar experimental setting, four different cell strains of fibroblasts were treated with 2000 U of IFN-γ for 48 hr and the conditioned medium was collected and evaluated for either tryptophan (**Panel B**) or kynurenine (**Panel C**) contents. Data is expressed as the mean +/- SD for four separate experiments. In another experiment, the relative levels of kynurenine in the conditioned medium collected from fibroblasts either untreated (squares) or treated (triangles) with 2000 U of IFN-γ for 48 hr and harvested at the indicated time-points post IFN-γ removal, were determined (**Panel D**). Data is shown as the mean +/- SD for three separate experiments. The asterisks denote a significant difference in either tryptophan or kynurenine levels between IFN-γ untreated (control) and treated fibroblasts (* $p<0.001$).

Figure 5.4. The proliferation of PBMC cocultured with IDO-expressing dermal fibroblasts is suppressed. Fibroblasts were treated with 2000 U of IFN-γ for 48 hr. To eliminate the effects of IFN-γ, conditioned medium was then replaced with fresh medium with no IFN-γ. IFN-γ-treated or –untreated (control) fibroblasts were then cocultured with isolated human PBMC for a period of 5 days. ^{3}H-thymidime was added to each coculture sample at a final concentration of 2 μCi/ml and 16 hr later, the floating PBMC were harvested, washed twice with PBS and their radioactive count was measured. In Panel A, PBMC were cultured either alone (**lane 1**), with non-IDO-expressing fibroblasts (**lane 2**), with non-IDO-expressing cells plus 200 μM of IDO inhibitor (1-methyl-D-tryptophan) (**lane 3**), or with IDO-expressing fibroblasts in the absence of IDO inhibitor (**lane 4**) or in presence of various concentrations of IDO inhibitor at final concentrations

of 50, 100, 200, 400 or 800 μM (**lanes 5-9**). Data represents the mean +/- SD for three separate experiments. The asterisks (*, ** $p<0.05$ and <0.001, respectively) denote a significant difference in proliferation of PMBC in control samples (cocoltured with non-IDO-expressing fibroblasts) and those either cocultured with IDO-expressing fibroblasts (lane 2 vs 4) or IDO-expressing cells in the presence of various concentrations of IDO inhibitor (lane 2 vs lanes 5, 6, 7 and 8). PBMC proliferation was restored at 800 μM concentration of 1-methyl-D-tryptophan (lane 9), where no significant difference was observed in the rate of PBMC proliferation between control and IDO-expressing fibroblasts (lane 2 vs 9).

Panel B, to evaluate the viability of PBMC, cells were stained with propydium iodide (PI) and the number of positive PBMC was measured in the samples cultured either alone (Panel Ba), cocultured with non-IDO-expressing fibroblasts (Panel Bb), or cocultured with IDO-expressing fibroblasts (Panel Bc) for 5 days.

Figure 6.4. IDO-expressing and non-IDO-expressing fibroblasts have a similar pattern of cell migration and proliferation. Fibroblasts populated in collagen gel were treated with either nothing (control) or 2000 U of IFN-γ (IDO-expressing) for 2 days. Conditioned medium was then replaced with fresh medium (with no IFN-γ). 6 mm punch biopsies were taken from each gel and placed onto six-well plates (3 biopsies per each well). The pattern of migrating cells were microscopically photographed at days 2, 4, 6 and 8 (**Panel A**). At the end of each time-point, punch biopsies were removed, cells were harvested and fibroblast proliferation assay was performed (**Panel B**). Solid and open bars represent IDO-expressing and non-IDO-expressing fibroblasts, respectively.

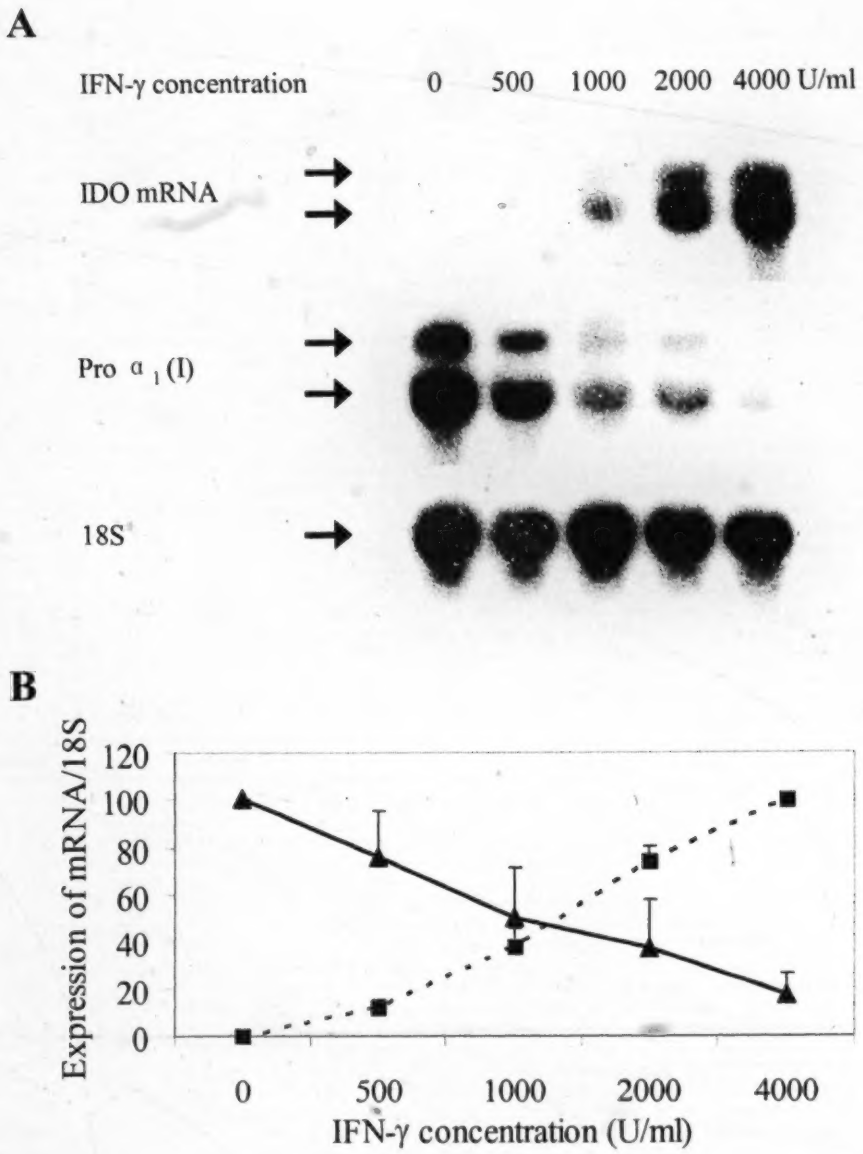
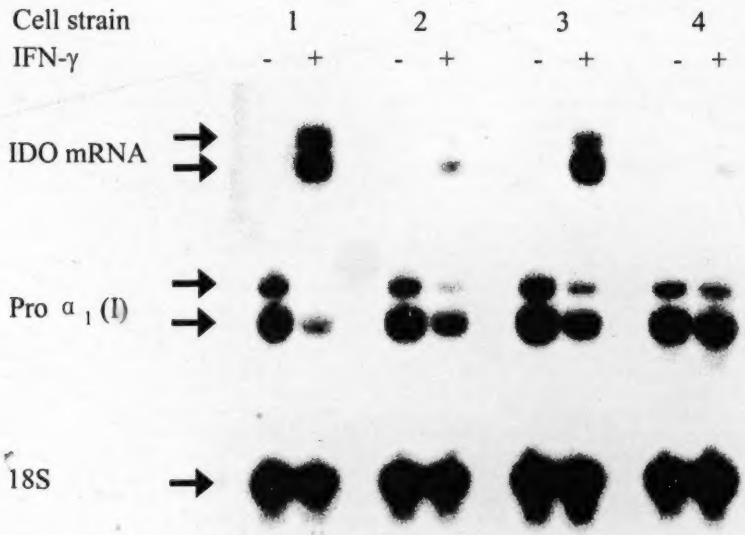

A
IFN-γ concentration
0
500
1000
2000
4000 U/ml
IDO mRNA
Pro α 1 (I)
18S
B
Expression of mRNA/18S
120
100
80
60
40
20
0
0
500
1000
2000
4000
IFN-γ concentration (U/ml)

Figure 1.4

A


Cell strain
1
2
3
4
IFN-γ
- + - + - + - +
IDO mRNA
Pro α 1 (I)
18S

B

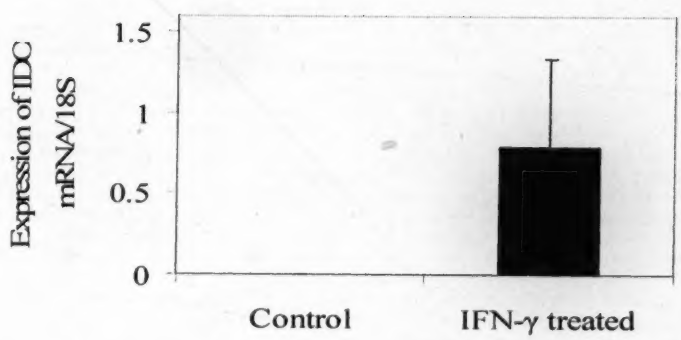

Expression of IDC mRNA/18S
1.5
1
0.5
0
Control
IFN-γ treated

Figure 2.4

C

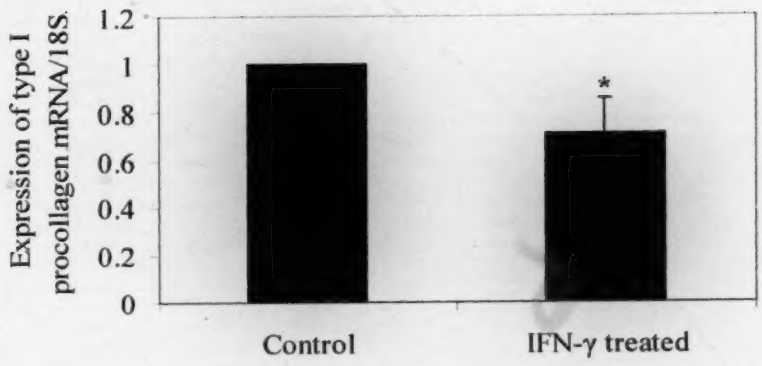

Expression of type I procollagen mRNA/18S.
1.2
1
0.8
0.6
0.4
0.2
0
*
Control
IFN-γ treated

Figure 2.4

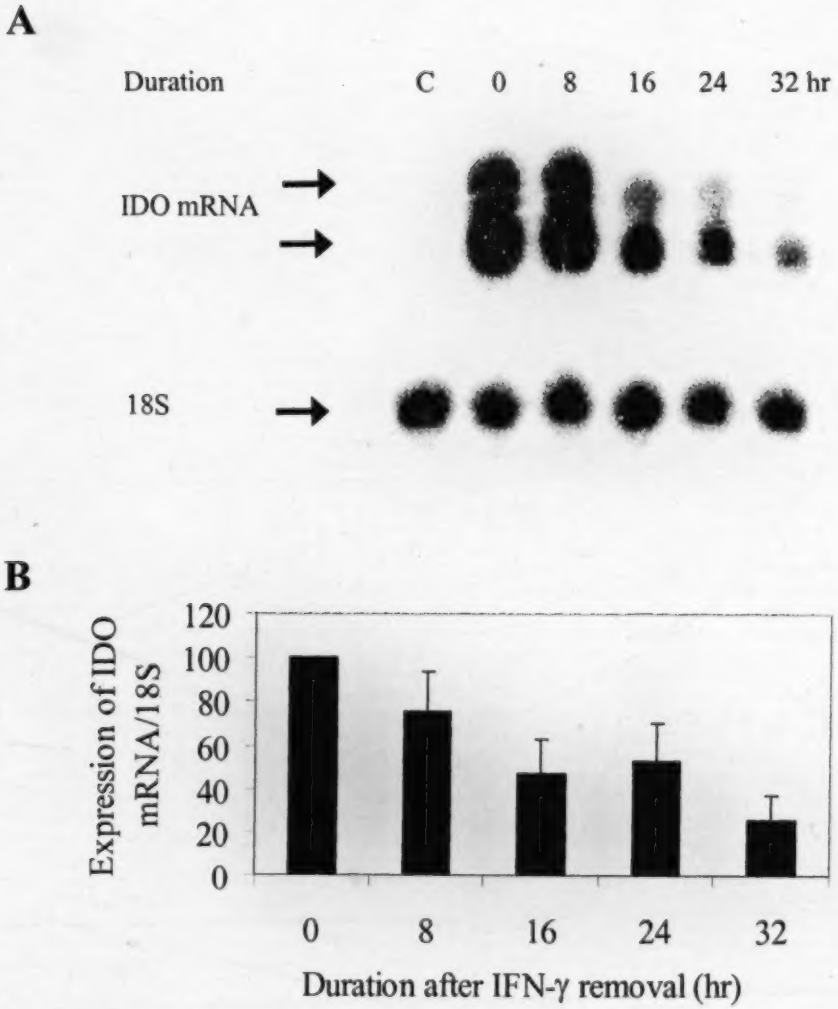
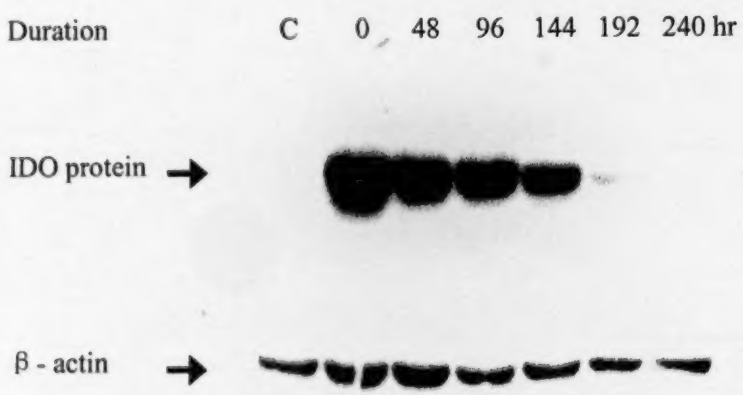

A
Duration
C
0
8
16
24
32 hr
IDO mRNA
18S
B
Expression of IDO mRNA/18S
120
100
80
60
40
20
0
0
8
16
24
32
Duration after IFN-γ removal (hr)

Figure 3.4

C


Duration
C
0
48
96
144
192
240 hr
IDO protein
β - actin

D

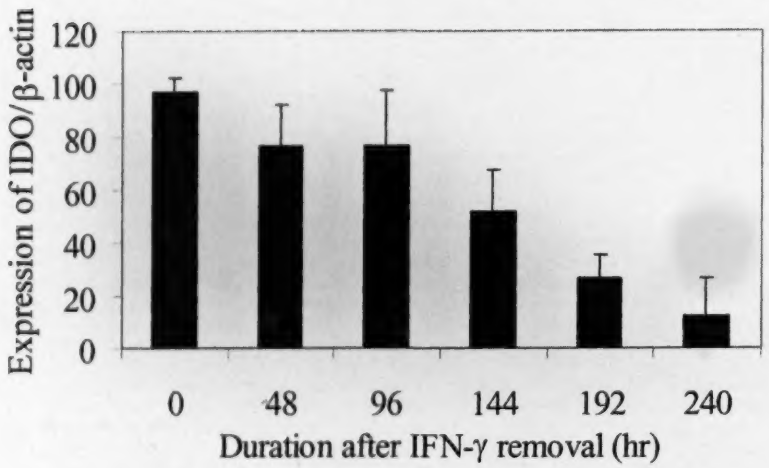
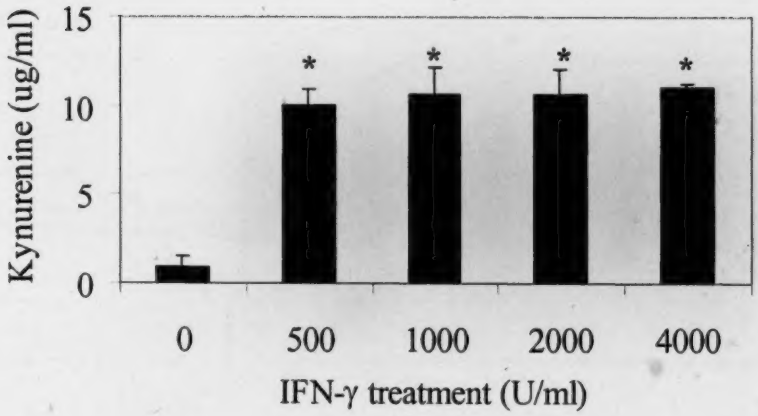

Expression of IDO/β-actin
120
100
80
60
40
20
0
0
48
96
144
192
240
Duration after IFN-γ removal (hr)

Figure 3.4

A


Kynurenine (ug/ml)
15
10
5
0
*
*
*
*
0
500
1000
2000
4000
IFN-γ treatment (U/ml)

B

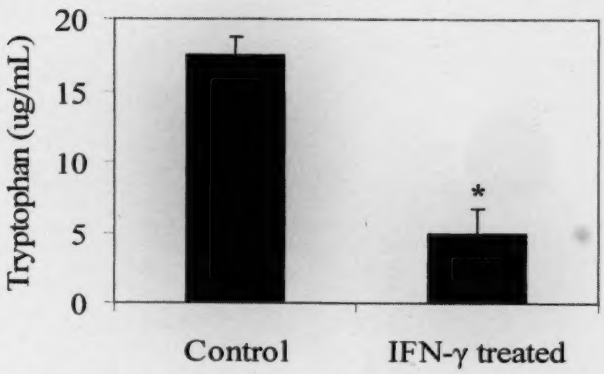
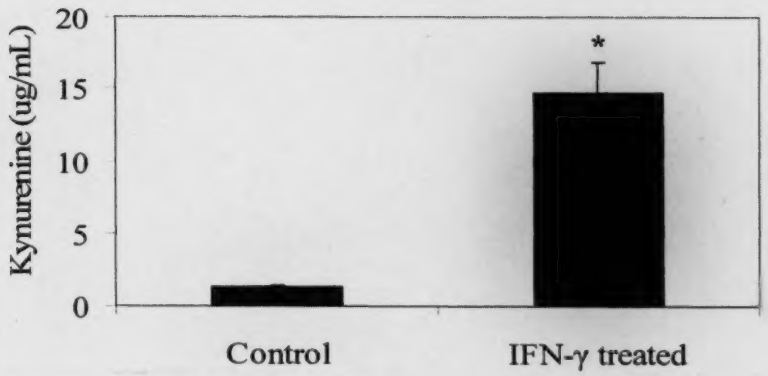

Tryptophan (ug/mL)
20
15
10
5
0
*
Control
IFN-γ treated

Figure 4.4

C


Kynurenine (ug/mL)
20
15
10
5
0
*
Control
IFN-γ treated

D

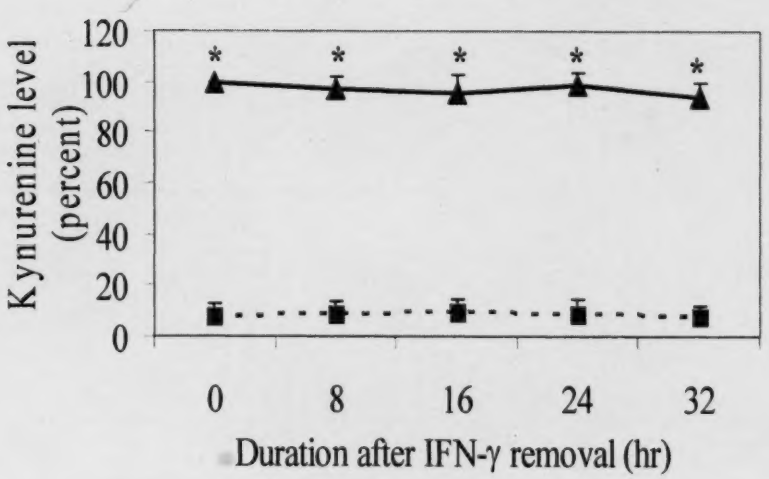
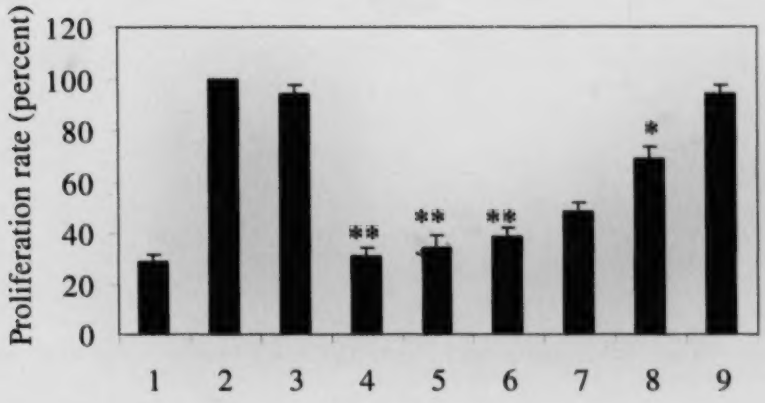

Kynurenine level (percent)
120
100
80
60
40
20
0
*
*
*
*
*
0
8
16
24
32
Duration after IFN-γ removal (hr)

Figure 4.4

A


Proliferation rate (percent)
120
100
80
60
40
20
0
**
**
**
*
1
2
3
4
5
6
7
8
9

B

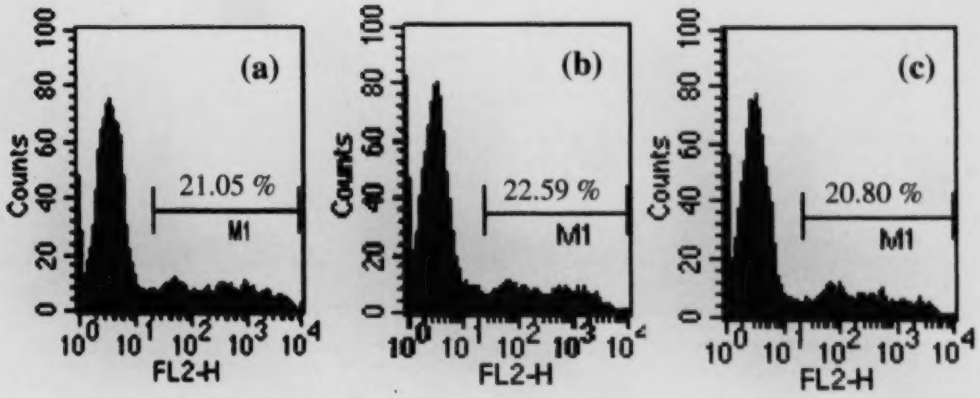

(a)
(b)
(c)
Counts
21.05 %
22.59 %
20.80 %
M1
FL2-H

Figure 5.4

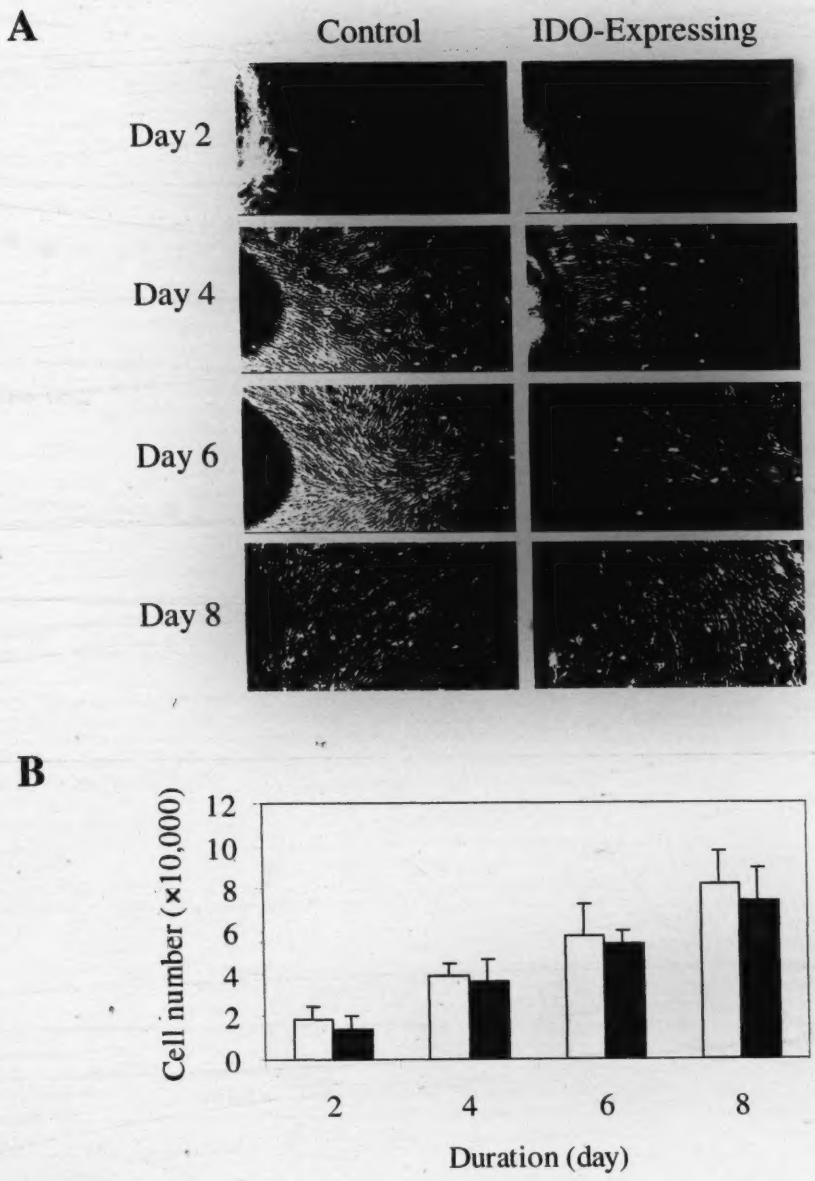

A
Control
IDO-Expressing
Day 2
Day 4
Day 6
Day 8
B
Cell number (×10,000)
12
10
8
6
4
2
0
2
4
6
8
Duration (day)

Figure 6.4

CHAPTER FIVE:

DEVELOPMENT OF AN IDO-EXPRESSING NON-REJECTABLE SKIN SUBSTITUTE OF FIBROBLASTS POPULATED IN COLLAGEN GEL (FPCG) UTILIZING A TEMPERATURE-SENSITIVE POLYMER

ABSTRACT

Indoleamine 2,3-dioxygenase (IDO) is an intracellular tryptophan-catabolizing enzyme possessing various immunosuppressive properties. Here, we report the use of this enzyme to suppress the proliferation of peripheral blood mononuclear cells (PBMC) cocultured with IDO-expressing fibroblasts of an allogeneic skin substitute *in vitro*. Fetal foreskin fibroblasts populated within collagen gel (FPCG) were treated with interferon-gamma (IFN-γ) conjugated with a temperature-sensitive polymer to induce the expression of IDO mRNA and protein. SDS-PAGE showed successful conjugation of IFN-γ with the temperature-sensitive polymer. Expression of IDO mRNA was evaluated by Northern analysis. IDO enzyme activity was evaluated by the measurement of kynurenine levels. The results of Northern blot analysis showed an induction of IDO mRNA expression when treated with polymer-conjugated IFN-γ. Kynurenine levels, as a measure of IDO bioactivity, were significantly higher in IFN-γ-treated fibroblasts, than in controls ($p<0.001$). In a lasting effect experiment, the expression of IDO mRNA in FPCG treated with polymer-conjugated IFN-γ was significantly longer than in those treated with free (non-conjugated) IFN-γ ($p<0.001$). IFN-γ radiolabeling showed a prolonged retention of IFN-γ within collagen gel in its polymer-conjugated form, compared to its free form. Presence of IDO protein in FPCG was demonstrated by western analysis even 16 d after removal of the conditioned medium (containing released IFN-γ). To demonstrate the immunosuppressive effects of IDO on the proliferation of PBMC, IDO-expressing FPCG treated with polymer-conjugated IFN-γ were cocultured with PBMC for a period of 5 days. The results showed a significant reduction in proliferation of PBMC cocultured with IFN-γ-treated IDO-expressing fibroblasts, compared to when cocultured with non-IDO-

expressing fibroblasts ($p<0.001$). The addition of an IDO inhibitor (1-methyl-D-tryptophan) reversed the suppressive effects of IDO on PBMC proliferation.

The effect of IDO-expressing FPCG on wound closure was evaluated *in vivo* and the results showed an accelerated rate of wound closure in wounds covered with FPCG when compared to controls. Hematoxylin & Eosin (H&E) staining and IDO immunohistochemical analysis showed the presence of intact FPCG and IDO protein at the wound site even at day 8 post wounding.

In conclusion, IDO expression in FPCG suppresses the proliferation of immune cells *in vitro*. The use of a temperature-sensitive polymer further prolongs the effect of IFN-γ on the expression of IDO. As well, the use of the skin substitute made up of FPCG facilitates wound closure *in vivo*. Therefore, modulating IDO levels *in situ* might be an alternative for prolonging the survival of skin allografts.

INTRODUCTION

Extensive skin loss from severe thermal injury causes significant functional morbidity and mortality (Cairns *et al,* 1993) and is considered a major health hazard to adults and children (Linares *et al*, 1990). The annual number of burn incidents in the United States is reported to be approximately 1.25 million (Brigham *et al*, 1996) from which 60,000 to 80,000 people are hospitalized (Boyce, 2001). In recent years, however, the overall mortality rate has improved for patients who suffer from burns, due in part to the significant biotechnological advancements in skin replacement for wound closure (Cairns *et al,* 1993). Rheinwald and Green (1975) initially introduced the *in vitro* cultivation of keratinocytes with the support of a feeder layer of lethally irradiated 3T3-cells. These investigators were able to grow keratinocytes to a confluency suitable for grafting (Gallico *et al,* 1984).

However, the procedure of expanding skin is not practical for some patients such as those with a large injury. It takes 4-6 weeks for autologous skin cells to become available for transplantation and leaving the wound open for this period increases the chances of heat and fluid loss, as well as bacterial infections. Also, patients with large wounds usually do not have enough uninjured skin available for cell culture. Furthermore, donor sites have a tendency to develop dermal fibroproliferative complications such as hypertrophic scar and keloid tissue. Finally, sheets of autologous keratinocytes grown alone are very fragile and difficult to handle. The best solution is to prepare an allogeneic skin substitute, which could be made available immediately. Considerations must also be made for the immunogenicity of skin cells, so that allograft is not rejected. Therefore, the purpose of this study is to evaluate the feasibility of

utilizing temperature-sensitive polymers to locally deliver IFN-γ as a means to induce the expression of indoleamine 2,3-dioxygenase (IDO) in fibroblasts populated in collagen gel (FPCG) and study the effect of IDO expression by FPCG on proliferation of allogeneic immune cells.

Interferon-gamma (IFN-γ), a pleiotropic cytokine, is an important component of the immune system. It is produced mainly by activated T helper cells (Th1) (Mosmann *et al,* 1989), natural killer (NK) cells (Perussia, 1991), and $CD8^+$ cytotoxic cells (Sad *et al,* 1995). IFN-γ exerts its effects by binding to specific receptors on human fibroblasts (Anderson *et al,* 1982), which then activate the Jak-STAT signal transduction pathway (Sadir *et al,* 2000). IFN-γ has been shown to be a potent inducer of IDO (Takikawa *et al,* 1988) and has therefore been used to induce IDO production in fibroblasts.

Indoleamine 2,3-dioxygenase (IDO) is a monomeric enzyme which catalyzes the rate-limiting step in the conversion of tryptophan, the least available essential amino acid in the body, into kynurenine (Takikawa *et al,* 1988). It has been suggested that induction of IDO is important in the host cell defense mechanisms especially in pathological conditions (Hiroaki *et al,* 1996), intracellular parasite infection (Pfefferkorn, 1984) and tumor cell growth (Gresser, 1989). The anti-parasitic and anti-tumor activities of IDO have been shown to be due to a tryptophan deficiency (Taylor *et al,* 1991). This suggests a mechanism by which IDO exerts its protective effects. The immunosuppressive properties of IDO have been recently investigated. Munn *et al.* (1998) showed that the expression of IDO can protect allogeneic fetuses from the mother's immune system. In addition, IDO expression by different cell types such as macrophages (Munn *et al,* 1999), dendritic cells (Hwu *et al,* 2000) and natural killer cells (Frumento *et al,* 2002) has been

shown to inhibit T cell functions, suggesting a mechanism through which immune responses are inhibited.

Temperature-sensitive polymers are polymers that possess temperature-dependent solubility. Due to this unique characteristic, these polymers are being explored by the pharmaceutical industry for the controlled delivery of drugs (Ron *et al,* 1998). They have also been used in biological systems locally for the delivery of anti-thrombogenic agents on blood-contacting surfaces (Gutowska *et al,* 1995). N-isopropylacrylamide (NiPAM) polymers are a group of thermoreversible polymers which have been studied extensively. The incorporation of succinimide esters in these polymers allows them to be conjugated to the amine groups of proteins without additional cross-linking agents (Uludag *et al,* 2001). In physiological conditions, thermoreversible polymers are in their insoluble form because their lower critical solution temperature (LCST: temperature for soluble ↔ insoluble transition) is typically around 30°C.

We have shown previously shown the immunosuppressive effects of IDO on the proliferation of peripheral blood mononuclear cells (PBMC) (Sarkhosh *et al,* 2003a; Sarkhosh *et al,* 2003b). However, the persistence of IDO expression following IFN-γ removal was relatively short. The purpose of this study is, therefore, to prolong the expression of IDO by delivering IFN-γ in a conjugated form with a temperature-sensitive polymer. We hypothesize that conjugation of IFN-γ with the temperature-sensitive polymer can prolong the expression of IDO. Our findings reveal that polymer-conjugated IFN-γ induces and prolongs the expression of IDO mRNA and its enzyme activity in the FPCG. Furthermore, IDO production generates a tryptophan-deficient microenvironment *in vitro* in which human PBMC are unable to proliferate.

MATERIALS AND METHODS

Fibroblast cell culture:

Cultures of human foreskin fibroblasts were established as described previously by Ghahary *et al.* (1997). In brief, punch biopsy samples were prepared from human foreskin. The tissue was collected in DMEM with 10% FBS (GIBCO, Grand Island, NY), minced into small pieces of less than 0.5 mm in any dimension, washed with sterile medium six times, and distributed into 60×15 mm Petri culture dishes (Corning Inc., Corning, NY) with four pieces per dish. A sterile glass coverslip was attached to the dish with a drop of sterile silicone grease to immobilize the tissue fragment. DMEM + antibiotics (penicillin G sodium 100 U/ml, streptomycin sulfate 100 μg/ml, and amphotericin B 0.25 μg/ml) with 10% FBS was added to each dish and incubated at 37°C in a water-jacketed humidified incubator in an atmosphere of 5% CO_2. The medium was replaced twice weekly. After 4 weeks of incubation, cells were released from dishes by brief (5 min) treatment with 0.1% trypsin (Life technologies Inc., Gaithersburg, MD) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) in PBS (pH 7.4) and transferred to 75 cm^2 culture flasks (Corning Inc., Corning, NY). Thereafter, once visual confluence was reached, cells were subcultured 1:6 by trypsinization. Fibroblasts from passages 3 to 7 were used for this study.

Temperature-sensitive polymer preparation:

Preparation of N-isopropylacrylamide (NiPAM) homopolymer and NiPAM copolymer containing N-acryloxysuccinimide (NASI) was reported previously by Uludag

et al. (2000). Briefly, NiPAM and NASI at desired amounts were dissolved in 25 ml of dioxane, followed by addition of 0.023g of BPO initiator. The solution was purged with N_2 for 30 min while stirring with a magnetic stirrer. After incubation at 70°C for 22 hr, polymers were precipitated by hexane, purified by dissolving the precipitate in THF and precipitated again by diethyl ether. Polymers were then dried at 50°C under a vacuum for one week. The composition of the synthesized polymers was determined by proton Nuclear Magnetic Resonance (^{1}H-NMR), using the chemical shifts (δ) for NiPAM and NASI (3.9 ppm and 2.8 ppm, respectively) to normalize them for the number of protons present in each peak. A spectroscopic method was used also to determine the NASI content of the polymers. The composition of the polymer used was NiPAM:NASI at 94.95: 5.05 mol % ratio. The molecular weight of the polymer was approximately 183kDa, as measured by light scattering (Uludag *et al,* 2001).

SDS-PAGE:

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on IFN-γ-polymer samples to ensure the successful conjugation of IFN-γ with temperature-sensitive polymers. Briefly, 2000 U of IFN-γ was added to 40 μl of temperature sensitive polymer (40 mg/ml) and incubated at 4 °C for overnight. Equal amounts of IFN-γ were then run on SDS-PAGE. The bands were visualized by 0.25% Coumassie Brilliant Blue staining.

Effect of polymer treatment on fibroblast proliferation:

Fibroblast proliferation assay was performed on the cells seeded in six-well plates at 1×10^5/well. Fibroblasts were either treated with 20 mg/ml of temperature-sensitive polymer or nothing (control) for different durations of time. They were then detached by brief (5 min) treatment with 0.1% trypsin (Life technologies Inc., Gaithersburg, MD) and 0.02% EDTA (Sigma, St. Louis, MO) in PBS (pH 7.4), re-suspended in 1 ml of DMEM with 10% FBS (GIBCO, Grand Island, NY) and counted using a hemacytometer.

Fibroblasts populated in collagen gel (FPCG) and IFN-γ treatment:

FPCGs were prepared using bovine type I collagen that was extracted in our laboratory by a procedure of Volpin and Veis (1971), using modifications to the procedure as described by Bell *et al.* (1979) as described previously by Nedelec *et al.*(1995) . Experiments were performed using six-well plates (ICN Biomedicals Inc., Aurora, OH). Each treatment group was prepared in triplicate. To begin, the following were added to each well: 350 µl 3×DMEM + Antibiotics, 26 µl 0.4 N NaOH, 440 µl cell suspension (2×10^6 cells/ml) in 1×DMEM + 10% FBS, 40 µl IFN-γ-conjugated polymer (for a final concentration of 2000 U/ml), 125 µl FBS (for a final concentration of 10%) and 870 µl of acid-extracted fetal bovine type I procollagen (2.14 mg/ml). The controls were FPCG treated with either nothing, polymer alone or IFN-γ alone. After treatment, plates were transferred immediately to a humidified incubator at 37°C in an atmosphere of 5% CO_2 for 3 hr for collagen gel lattices to be formed. After this period, collagen gels were released from the plates using surgical blade (Fisher Scientific, Edmonton, AB). Human recombinant IFN-γ (Sigma, St. Louis, MO) was used for this study.

Northern blot analysis:

Following incubation for different times, FPCGs were harvested and collagen lattices were digested with bacterial collagenase (Sigma, St. Louis, MO) in PBS (4mg/10ml) at 37°C for 30 min. Isolated cells were pelleted by centrifugation at 1100 rpm for 10 min. Fibroblasts were then lysed with 500 µl of 4 M guanidium isothiocyanate (GITC) and the total RNA from each group was isolated by guanidium isothiocyanate/CsCl procedure using phenol:chloroform (1:1) followed by chloroform:isoamyl alcohol (49:1) as described previously by Chirgwin *et al.* (1979). Total RNA from each individual fibroblast culture was then separated by electrophoresis (10 µg per lane) on a 1% agarose gel containing 2.2 M formaldehyde. Subsequently, it blotted onto nitrocellulose paper. To control the RNA loading, quantities of 18S ribosomal RNA were compared visually by ethidium bromide fluorescence. The nitrocellulose blots were baked for 2 hr at 80°C under a vacuum and prehybridized for 4 hr at 45°C in a prehybridization solution. Hybridization was performed at 45°C in the same solution using either IDO or 18S cDNA. The cDNA probes were labeled with P-α^{32}-dCTP by nick translation. Filters were washed with 2×sodium citrate/sodium chloride buffer and 0.1% sodium dodecylsulfate for 1 hr at room temperature. They were washed again with 0.1×sodium citrate/sodium chloride buffer and 0.1% sodium dodecylsulfate for 20 min at 65°C. Autoradiography was performed by exposing Kodak X-Omat film to nitrocellulose filters at -80°C in the presence of an intensifying screen. Quantitative analysis of autoradiographs was accomplished by densitometry. The cDNA probe for 18S was obtained from the American Type Culture Collection

(Rockville, MD). The IDO cDNA was a generous gift from Dr. J.M. Carlin (Department of Microbiology, Miami University, USA).

Determination of total kynurenine in the conditioned medium:

The biological activity of IDO was evaluated by measuring the level of kynurenine, a degradation product of tryptophan oxidation that was present in conditioned media derived from IFN-γ-treated fibroblasts. Briefly, 10 μl of internal standard (homophenyl alanine) was added to 50 μl of the cell culture medium. Conditioned medium was then deproteinized by adding 100 μl of acetone. Next, samples were vortexed, cooled on ice and centrifuged at 13,000 rpm for 15 min at 4°C. Subsequently, 10 μl of 0.25 M $HClO_4$ was added to the supernatant. The mixture was incubated at 25°C for 25 min and then evaporated to dryness under a vacuum. 500 μl of ethanol-acetyl chloride reagent was added to the residue in a screw-cap glass tube and heated at 110°C for 30 min. The solution was again evaporated to dryness under a vacuum. The residue was dissolved in 40 μl of 20% MeOH in water and analyzed by liquid chromatography with electrospray mass spectrometry (LC/EMS), using a Hewlett Packard series 1100 mass selective detector controlled by 1100-MED Chem Station. The mass spectrometer was operated in the positive-ion mode. Kynurenine quantitative analysis was performed in the selected monitoring ion-mode.

^{125}I-labeling:

The rate of IFN-γ release from collagen gels in their polymer-conjugated or free forms was studied *in vitro* using ^{125}I-labeled IFN-γ and the iodination method of Fraker

and Spack (1978). Briefly, 1.5 ml centrifuge tubes were coated with 200μl of 20μg/ml Iodogen (1,3,4,6-tetrachloro -3α, 6α-diphenylglycouril, TCDG)(Sigma, St. Louis, MO) which was allowed to dry overnight. Next, 5 μg of IFN-γ and 0.5 mCi of free ^{125}I (5μl) were added to the tube and the volume was brought to 50μl using 100 mM phosphate buffer. After 25 min, the labeled protein was purified by elution through a NAP-5 column (Pharmacia, Piscataway, NJ) with 100mM phosphate buffer. Trichloroacetic acid precipitation of the purified fraction indicated that >95% of protein was bound to ^{125}I.

Western blot analysis:

Western blot analysis was performed on harvested FPCGs at specified time-points after the removal of conditioned medium. Cell extracts were prepared by adding 1×10^6 fibroblasts to lysate buffer (50 mM Tris-HCl [pH=7.40], 150 mM NaCl, 1mM EDTA (Sigma, St. Louis, MO), 1mM EGTA (Sigma-Aldrich Canada, Ltd.), 0.025% NaN_3, 1% TritonX-100, 0.5% Igepal CA-630 (Sigma, St. Louis, MO), and protease inhibitor cocktail (Sigma, St. Louis, MO)). Extracts were centrifuged at 13,000 rpm for 10 min at 4°C and 50 μg (BSA protein assay [Pierce]) of the total protein was run on a 10% SDS-PAGE. Proteins were then transferred to a PVDF membrane (Millipore Corp., Bedford, MA) by a Mini Trans-Blot Cell (Bio-Rad Laboratories Ltd., Mississauga, ON). Blots were incubated with IDO polyclonal antibody (prepared in our laboratory). Horseradish peroxidase (HRP)-conjugated secondary antibody (Sigma, St. Louis, MO) was used and then visualized using the ECL+Plus™ western blotting detection system (Amersham Biosciences).

Peripheral blood mononuclear cell (PBMC) proliferation by ^{3}H-thymidine incorporation assay:

Samples of 25 to 30 ml of human blood were drawn into heparinized tubes from normal individuals. Total PBMC was isolated by density gradient sedimentation on Histopaque 1077 (Sigma, St. Louis, MO) following the manufacturer's protocol. Briefly, whole blood was layered onto an equal volume of Histopaque and centrifuged at 2000 rpm for 20 min at room temperature. PBMC were then isolated, added to RPMI 1640 + 10% FBS (GIBCO, Grand Island, NY), pelleted by centrifugation at 2000 rpm for 10 min and were washed twice with RPMI 1640. For the mixed lymphocyte reaction assay, 2×10^{5} fibroblasts were embedded within collagen gel, and treated with polymer-conjugated IFN-γ for 48 hr at a concentration of 2000 U/ml in twelve-well plates (Costar, Cambridge, MA). Subsequently, 1×10^{4} fibroblasts were seeded onto the collagen gel in a monolayer to activate the proliferation of allogeneic PBMC. Fibroblasts were then irradiated with 2000 units of gamma rays and cocultured with 4×10^{5} of PBMC in RPMI 1640 + 10% FBS for a period of 5 days. To demonstrate the specificity of IDO action on PBMC proliferation, an IDO inhibitor, 1-methyl-D-tryptophan (Aldrich Chem. Co., Milwaukee, WI), was added to coculture samples at a final concentration of 800 μM.

To measure the rate of PBMC proliferation in response to exposure to allogeneic fibroblasts, a ^{3}H-thymidine proliferation assay was performed on PBMC cocultured with FPCG. ^{3}H-thymidine (PerkinElmer Life Sciences Inc., Boston, MA) was added to the conditioned medium of each sample for a final concentration of 2 μCi/ml and was incubated for 16 hr. After this period, PBMC were harvested and the collagen lattice was digested using collagenase (4 mg/ 10 ml). PBMC were then washed twice with PBS,

dissolved in GITC and added to scintillation fluid (Amersham Canada Ltd., Oakville, ON). Radioactive counting was performed using the scintillation counter (Beckman) and measured in counts per min (cpm).

Animal model:

The use of animals in our study was approved by the University of Alberta Health Sciences Laboratory Animal Services (HSLAS). Full-thickness wounds were created in two rows on the rat dorsum (three wounds per row) using a 6 mm punch biopsy (Acuderm Inc., Ft. Lauderdale, FL), while rats were under isoflurane (Biomedia-MTC; Animal health Inc., Cambridge, ON) inhalation anesthesia. The wounds were then immediately covered with either nothing, collagen alone, IFN-γ (conjugated with temperature-sensitive)-treated IDO-expressing FPCG or non-IDO-expressing FPCG. Using an approved window wrapping technique, wounds were covered for 4 days with a dental dam (non-flavored) using bandage tapes (Johnson & Johnson). Every 24 hr after wounding, all wounds were dressed with Phlogel Ultra (J.A.R. Pharmaceuticals Ltd., Edmonton, AB).

Localization of IDO in FPCGs placed on wounds:

Six-micrometer thick cryosections made for immunostaining were placed on poly-L-lysine-coated slides and fixed with acetone. Tissues were incubated with rabbit polyclonal IDO antibody (prepared in our laboratory) overnight at a concentration of 1:1000 at room temperature. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma) was used as a secondary antibody. Signal detection was carried out using a 3,3'-

diaminobenzidine (DAB) staining kit (Dako Diagnostics, ON, Canada). Slides were subsequently counterstained with hematoxylin.

RESULTS

Temperature-sensitive polymer can be conjugated to IFN-γ:

SDS-PAGE results on IFN-γ samples following overnight incubation with temperature-sensitive polymer show a disappearance of IFN-γ band, since large molecular weight of the polymer (183 KDa) does not allow penetration of the IFN-γ into the gel. Addition of a conjugation competitive inhibitor NH_4OH to the IFN-γ-polymer mixture causes a reappearance of IFN-γ band, which shows the specificity of the polymer conjugation (Fig. 1.5A).

Fibroblast proliferation is not influenced by temperature-sensitive polymer:

To assess the effect of temperature-sensitive polymer on fibroblast proliferation, fibroblast proliferation assay was performed on the cells treated with temperature-sensitive polymer for different durations (Fig. 1.5B). A comparison between polymer-treated and -untreated fibroblasts at days 1, 3, and 5 post-treatment does not reveal a significant difference in fibroblast proliferation (n=3).

Polymer-conjugated IFN-γ induces the expression of IDO mRNA in FPCG:

To evaluate whether IDO mRNA expression is inducible in FPCG upon treatment with IFN-γ, Northern blot analysis was performed on the total RNA extracted from fibroblasts treated with polymer-conjugated IFN-γ (2000 U/ml) or untreated (control) for

48 hr (Fig. 2.5A). IDO mRNA expression is inducible in FPCG upon treatment with 2000 U of IFN-γ (in both free and conjugated forms) for 48 hr. Densitometry results of three separate experiments are also combined (Fig. 2.5B), which reveal that the level of IDO mRNA expression is significantly increased in the FPCG following treatment with both free and polymer-conjugated forms of IFN-γ ($p<0.001$, $n=3$).

IDO expression is prolonged in FPCG following treatment with polymer-conjugated IFN-γ:

To assess whether IFN-γ conjugation with temperature-sensitive polymer prolongs the expression of IDO mRNA in fibroblasts, FPCG were treated with 2000 U of IFN-γ for 48 hr after which the conditioned medium (containing released IFN-γ) was removed, FPCG were washed twice with PBS and fresh medium (with no IFN-γ) was added. Fibroblasts were harvested at days 0, 2, 4, and 6 after removal of conditioned medium and subjected to Northern analysis. Representative blot is shown (Fig. 3.5A). Results show that the expression of IDO mRNA is prolonged in the FPCG treated with conjugated form of IFN-γ compared to those treated with free form of IFN-γ. To statistically analyze the data, densitometry results from three separate experiments on the expression of IDO mRNA are also combined (Fig. 3.5B). Results reveal a significant difference in the level of IDO mRNA expression in FPCG treated with polymer-conjugated and free IFN-γ (0.78 ± 0.08 and 0.32 ± 0.12, respectively) at day 4 following conditioned medium removal ($p<0.001$, $n=3$). To investigate this prolongation of IDO production at protein level, in a similar experimental setting, western blot was performed on the FPCG treated with polymer-conjugated IFN-γ at days 0, 4, 8, 12, and 16 following

conditioned medium removal (Fig. 3.5C(a)). Results show presence of IDO protein in FPCG even 16 days post conditioned medium removal. Coumassie brilliant blue staining was performed on the same blot to show the amount of protein loading (Fig. 3.5C(b)). Furthermore, to investigate release of polymer-conjugated IFN-γ from the collagen gel, release of a radiolabeled IFN-γ (in both polymer-conjugated and free forms) from the collagen gel to conditioned medium was studied following incubation for different times (Fig. 3.5D). Results show a sharp release of free IFN-γ into the conditioned medium following 2 days of incubation (60 ± 7%), compared to the release of polymer-conjugated IFN-γ (21 ± 2%). Following 6 days of incubation, most of the free IFN-γ is released from the collagen gel (97 ± 5%), compared to polymer-conjugated IFN-γ (45 ± 4%). The free IFN-γ is almost completely released from the collagen gel following 8 days of incubation (100 ± 0 %), compared to the polymer-conjugated IFN-γ (58 ± 4%).

Kynurenine levels are increased in FPCG treated with IFN-γ:

To measure the enzyme activity of IDO in FPCG, kynurenine levels were measured in conditioned media of IFN-γ untreated (control) and treated (polymer-conjugated and free) fibroblasts (Fig. 4.5A). Kynurenine levels are significantly increased in the FPCG treated with 2000 U of IFN-γ in its either free or polymer-conjugated form (4.2 ± 0.8 and 4.4 ± 0.7 μg/ml, respectively), compared to the controls of either untreated or treated with polymer alone (0.6 ± 0.5 and 0.5 ± 0.7 μg/ml, respectively) ($p < 0.001$, $n=3$).

Kynurenine levels were evaluated in the conditioned medium of FPCG treated with free and polymer-conjugated forms of IFN-γ for 48 hr (day 0) and replaced with

fresh medium (containing no IFN-γ) at days 2, 4 and 6 post conditioned medium (containing released IFN-γ) removal (Fig. 4.5B). Kynurenine production is significantly increased in the IFN-γ treated samples, compared to the controls ($p<0.001$, n=3). The level of kynurenine production is significantly reduced in conditioned medium of FPCG treated with free form of IFN-γ at day 6 following conditioned medium removal, compared to that in FPCG treated with polymer-conjugated IFN-γ at the same time-point ($p<0.05$, n=3).

PBMC proliferation is suppressed by IFN-γ-treated IDO-expressing FPCG in a co-culture system:

To measure the rate of PBMC proliferation following 5 days of incubation with IFN-γ-treated IDO-expressing fibroblasts, ^{3}H-thymidine incorporation assay was performed on either IFN-γ-treated or -untreated (control) FPCG. Results show that PBMC proliferation is significantly reduced when cocultured with IFN-γ-treated IDO-expressing FPCG (19,700 ± 3,300 cpm), compared to when cocultured with non-IDO-expressing (not IFN-γ treated) FPCG (59,900 ± 1,900 cpm). PBMC proliferation is not suppressed upon coculture with collagen alone in the presence of the fibroblast monolayer treated with polymer-conjugated IFN-γ (56,300 ± 3,400 cpm). Furthermore, addition of the IDO inhibitor (1-methyl-D-tryptophan) at a concentration of 800 μM reverses the inhibitory effects of IDO on PBMC proliferation (50,700 ± 3,200 cpm), suggesting the specificity of IDO action on proliferation of allogeneic PBMC ($p <0.001$, n=3) (Figure 5.5).

Wound closure is facilitated *in vivo* in wounds covered with FPCG:

To investigate the efficacy of the skin substitute made up of FPCG in an animal model, a pilot study was performed by placing the skin substitute on 6 mm punch biopsy excisional wounds for 8 days (Figure 6.5). Pictures of rats immediately (Panel Aa) and 8 days post wounding (Panel Ab) are shown. Relative wound sizes at days 0, 4 and 8 post-wounding were calculated by dividing the wound area at each time-point by the original wound area. A combined result of two separate experiments is depicted (Panel B). As shown, placing the FPCG (containing either non-IDO-expressing or IDO-expressing fibroblasts) on the wound reduces the relative wound size to 32 ± 6% and 24 ± 5%, respectively, compared with controls of being either untreated or treated with collagen alone (66.9 ± 6% and 61.0 ± 7%, respectively) at day 8 post-wounding ($p<0.001$, $n=2$). A cross-sectional (H&E) micrograph of the wound covered with either collagen alone (Panel C) or FPCG in absence (Panel D) and presence (Panel E) of polymer-conjugated IFN-γ is shown. Arrows indicate lateral extent of the wound, which show an intact skin substitute on the wounds covered with FPCG. It seems that epithelialization is faster in wounds covered with IDO-expressing FPCG, compared to those covered with non-IDO-expressing FPCG. Immunohistochemical staining of the IFN-γ-treated cross-section also shows the presence of IDO protein in wounds covered with IFN-γ-treated FPCG at day 8 post-wounding (Panel F).

DISCUSSION

The purpose of this study was to investigate the effects of IFN-γ-induced IDO expression in fibroblasts populated in collagen gel, by employing a temperature-sensitive

polymer to deliver IFN-γ locally. Immunosuppressive properties of IDO on the cocultured allogeneic PBMC and feasibility of utilizing an *in vivo* model in determining the efficacy of biological skin substitute were also evaluated. The expression of IDO was inducible in fibroblasts upon treatment with polymer-conjugated IFN-γ. This expression was prolonged when fibroblasts were treated with conjugated IFN-γ, as compared to free IFN-γ. Our study also showed that IDO expression in fibroblasts significantly suppressed the proliferation of PBMC *in vitro*. Our biological skin substitute also facilitated wound closure *in vivo*. Therefore, IDO may play an important role in constructing an allogeneic, non-rejectable and readily available skin substitute.

N-isopropylacrylamide (NiPAM)-based temperature-sensitive polymers were used because they possess several unique properties. They can be conjugated to proteins without the use of cross-linking agents and they are in a gel phase at physiological temperatures (Uludag et al, 2000). When these polymers were conjugated to bone morphogenetic protein 2 (BMP-2) and applied *in vivo*, they localized to the application site successfully and stimulated osteogenic processes (Gao *et al,* 2002). Thus, we hypothesized that the conjugation of IFN-γ with such polymers would also prolong the presence of IFN-γ in our skin substitute of FPCG. This in turn would prolong the expression of IDO in fibroblasts populated in collagen gel. Since IDO oxidizes tryptophan, the least available essential amino acid in the body, to kynurenine (Holmes, 1998), the expression of IDO generates a tryptophan-deficient microenvironment in which highly proliferating cells such as immune cells are unable to proliferate (Lee *et al,* 2002; Mellor *et al*, 2001; Mellor *et al,* 2002).

The lasting effect studies showed that IFN-γ prolongs the expression of IDO when it was conjugated with a temperature-sensitive polymer. The level of IDO mRNA was significantly higher after 6 days of conditioned medium removal in IFN-γ conjugated samples than in controls. Similar results were obtained in terms of IDO protein levels, which were still elevated after 16 days of conditioned medium removal. The same delay was observed in the level of kynurenine, final product of tryptophan oxidation, since it remained high in the IFN-γ-treated samples even six days after removal of the conditioned medium compared to the controls. Therefore, in IFN-γ-conjugated samples, IFN-γ is retained within collagen gels for a longer time periods. However, even with the polymer-conjugated samples, some release of IFN-γ was observed. This was likely due to sparingly soluble polymer slowly dissolved in the tissue culture medium. Furthermore, the results of ^{3}H thymidine incorporation assays showed that IDO expression in fibroblasts significantly reduces PBMC proliferation in a coculture system.

The effect of IFN-γ on expression of IDO is transient and decreases overtime after removal of the conditioned medium. Nevertheless, the expression of IDO in fibroblasts is likely long enough to delay T cell-mediated rejection. This would allow enough time for autogenic cells, such as keratinocytes, to migrate from the wound edges and form epidermal layer. The presence of keratinocytes induces a shift from a Th1 to a Th2 cytokine profile to induce tolerance in skin allografts containing a chimeric mixture of autogenic and allogeneic cells (Cao *et al*, 2003). Therefore, we suspect that IDO expression in our skin substitute may serve as a protecting factor in the initial time period by allowing the ingrowth of autogenic keratinocytes, and resulting in a local immune tolerance.

The use of IFN-γ as an inducer of IDO would be beneficial for burn patients to prolong graft survival. In a recent study, we have shown that burn patients have relatively lower levels of IFN-γ (unpublished data). Therefore, the use of IFN-γ may reduce fibroproliferative disorders commonly seen in these patients such as keloid and hypertrophic scarring. In addition, Halloran *et al.* (2001) showed that IFN-γ can prevent early thrombosis and necrosis in allograft tissue, which again would increase graft survival. We have recently also illustrated that IFN-γ and the expression of IDO in transfected keratinocytes downregulates the expression of MHC-I in these cells (unpublished data). Therefore, despite the known function of IFN-γ in upregulating MHC-I expression (Boehm *et al,* 1997), our finding suggests that expression of IDO can counteract this effect.

In regards to utilizing FPCG to facilitate the wound healing, several studies demonstrated that acellular dermis by itself is not an enough substrate to enhance keratinocyte growth in the artificial skin (Krejci *et al,* 1991; Shakespeare *et al,* 1987). In contrast, populating the skin substitute made up of acellular cadaver dermis with fibroblasts showed to facilitate keratinocyte growth and differentiation (Krejci *et al,* 1991). In fact, our results confirmed these findings, since application of the wound with collagen alone didn't accelerate the rate of wound closure. In addition, providing the dermal component of the skin substitute, in contrast to grafting cultured keratinocytes only, has been shown to reduce scarring and contraction (Langdon *et al,* 1988). Therefore, application of IFN-γ-treated FPCG would be beneficial in both accelerating the wound closure and reducing its contraction and scarring.

In conclusion, we have demonstrated that fibroblast treatment with the polymer-conjugated form of IFN-γ induced and prolonged the expression of IDO mRNA and protein in a three-dimensional cell culture system *in vitro*. The tryptophan-deficient microenvironment created by IDO inhibited immune cell proliferation. We also showed that the application of our cultured skin substitute accelerated the rate of wound healing *in vivo*. Our findings initiate a new approach by delivering therapeutic proteins to locally suppress immune cell proliferation. Therefore, IDO may be an important tool in developing a non-rejectable skin substitute to be used as wound coverage and a source of wound healing promoting factors.

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FIGURE LEGENDS

Figure 1.5. IFN-γ can be conjugated to the temperature-sensitive polymer and temperature-sensitive polymer does not influence fibroblast proliferation.

Temperature-sensitive polymer was conjugated to IFN-γ using the protocol described in the Methods. Following conjugation, samples of either IFN-γ alone (Lane 1), or IFN-γ and polymer in absence (Lane 2) or presence (Lane 3) of a conjugation inhibitor were run on a 10% SDS-PAGE (**Panel A**). To evaluate the effect of temperature-sensitive polymer on fibroblast proliferation, fibroblasts were treated with the temperature-sensitive polymer (20 mg/ml) for periods of 1, 3 and 5 days. A combined result of three separate experiments is shown (**Panel B**).

Figure 2.5. Expression of IDO mRNA is induced in FPCG upon treatment with polymer-conjugated IFN-γ.

FPCG were either untreated (Lane 1), or treated with polymer alone (Lane 2), 2000 U of IFN-γ in its free (Lane 3) and polymer-conjugated (Lane 4) forms for 48 hr. Following this period, total RNA was extracted from fibroblasts and subjected to Northern analysis for the expression of IDO mRNA and 18S. **Panel A**, representative Northern blot is shown. 18S rRNA is used as a loading control. A combined densitometry result of three separate experiments on the ratio of IDO mRNA/18S expression is also depicted (**Panel B**) (* $p<0.001$, $n=3$).

Figure 3.5. Expression of IDO is prolonged in FPCG upon conjugation of IFN-γ with the temperature-sensitive polymer.

FPCG were treated with 2000 U of IFN-γ both free and polymer-conjugated forms for 48 hr. Subsequently, conditioned medium (containing released IFN-γ from the collagen gel) was removed, FPCG were washed twice with PBS and fresh medium (with no IFN-γ) was added. Thereafter, conditioned medium was replaced with fresh medium every 48 hr. FPCG were harvested at the indicated time-points post conditioned medium removal. Northern blot analysis was performed on the expression of IDO mRNA in FPCG treated with either no agent (N), polymer alone (P), and IFN-γ in its polymer-conjugated (C) or free (U) form for 48 hr (Day 0) and at days 2, 4 and 6 post conditioned medium removal. Representative blot is shown (**Panel A**). 28S and 18S rRNA is used as a RNA loading control. Also, to statistically analyze the results, densitometry results on expression of IDO mRNA/18S obtained from three separate experiments are combined from FPCG treated with either free (solid bars) or polymer-conjugated (striped bars) IFN-γ (**Panel B**). The asterisks denote a significant decrease in the level of IDO mRNA expression in FPCG treated with free IFN-γ, compared to those treated with polymer-conjugated IFN-γ at days 4 and 6 post conditioned medium removal (* $p<0.001$, $n=3$).

FPCG were also treated with 2000 U of polymer-conjugated IFN-γ for 48 hr, after which the conditioned medium was removed, FPCG were washed with PBS and fresh medium (with no IFN-γ) was added. Fibroblasts were harvested at different time-points post conditioned medium removal and subjected to western analysis. A representative blot is shown (**Panel Ca**) in FPCG either untreated (C) or treated with 2000 U of conjugated IFN-γ and harvested at the indicated time-points post conditioned medium removal. Coomassie brilliant blue staining was performed on the same blot to show amount of the total protein, which serves as a loading control (**Panel Cb**). In a

separate experiment, the quantity of IFN-γ release from the collagen gel was studied. Either polymer-conjugated (■) or free (▲) IFN-γ was added to the collagen gel. Conditioned medium was harvested from the collagen gel at days 0, 2, 4, 6, 8, and 10 following addition of IFN-γ (**Panel D**) (n=4).

Figure 4.5. Kynurenine production is increased in the IFN-γ-treated FPCG.

FPCG were either untreated (control) or treated with 2000 U free or polymer-conjugated IFN-γ for 48 hr. Conditioned medium from FPCG was then harvested and analyzed for kynurenine levels, according to the procedure described in the Methods. **Panel A**, depicts the combined results of three separate experiments. Lanes 1-4 represent FPCG treated with no agent, polymer alone, free IFN-γ and polymer-conjugated IFN-γ, respectively (* $p<0.001$, n=3).

FPCG were also treated with 2000 U of IFN-γ in either free (solid black bars) or polymer-conjugated (dotted bars) forms for 48 hr. After this period, conditioned medium was harvested, FPCG were washed twice with PBS and fresh medium (with no IFN-γ) was added. Thereafter, conditioned medium was replaced with fresh medium every 2 days. Kynurenine analysis was performed on the collected conditioned medium after 48 hr of IFN-γ treatment (day 0) and at the indicated time-points post conditioned medium removal. A combined densitometry result of three separate experiments is shown (**Panel B**). The asterisk (* $p<0.001$) denotes a significant difference in the level of kynurenine production between FPCG treated with either free (solid black bars) or conjugated (dotted bars) IFN-γ either and the controls (C) of either untreated (solid bar) or treated with polymer alone (striped bar). The double asterisk (** $p<0.05$) shows a significant

difference in the level of kynurenine production between FPCG treated with free and polymer-conjugated IFN-γ at day 6 post conditioned medium removal.

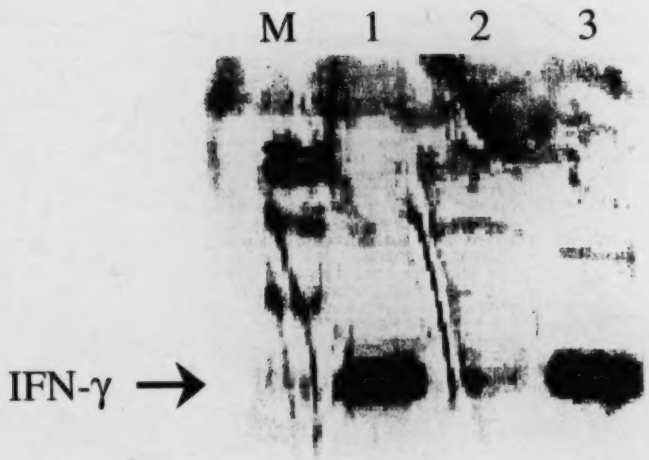
Figure 5.5. IDO expression in FPCG suppresses proliferation of PBMC in a coculture system.

FPCG were treated with 2000 U of polymer-conjugated IFN-γ for 48 hr and cocultured with PBMC for 5 days according to protocol described in the Methods. Subsequently, 2 μCi of ^{3}H-thymidime was added to each co-culture and incubated for 16 hr. Collagen gel was then dissolved using collagenase, PBMC were harvested and washed three times with PBS and incorporation of ^{3}H-thymidine into their cellular DNA was evaluated. PBMC were cultured either alone (Column 1), or co-cultured with non-IDO-expressing FPCG (Column 2), fibroblasts seeded on the collagen gel in presence of polymer-conjugated IFN-γ (column 4), or co-cultured with IDO-expressing FPCG in absence or presence of 800 μM IDO inhibitor (Columns 3 and 5, respectively). Data is presented as the mean +/- SD for 3 separate experiments. The asterisk (* $p<0.001$) denotes a significant difference in proliferation of PMBC co-cultured with IDO-expressing FPCG, compared to those co-cultured with non-IDO-expressing FPCG (Columns 3 and 2), as well as a significant difference in proliferation of PMBC co-cultured with IDO-expressing FPCG, in absence or presence of IDO inhibitor (Columns 3 and 5).

Figure 6.5. Application of FPCGs facilitates wound closure *in vivo*.

Full-thickness wounds were created on the rats' dorsum using 6 mm punch biopsy (**Panel Aa**) and immediately covered with different treatments. **Panel Ab**, shows the picture of the wounds at day 8 post wounding treated with collagen alone (Lane 1), no agent (Lane 2), and either IDO-expressing or non-IDO-expressing FPCG (Lanes 3 and 4, respectively). **Panel B**, illustrates the combined results of two separate experiments, showing the relative wound size at days 0, 4 and 8 post wounding in the wounds covered with no treatment (■), collagen alone (□), IFN-γ-treated IDO-expressing (○) and non-IDO-expressing (●) FPCG. Asterisk denotes a significant difference in wound size between wounds covered with either nothing or collagen alone and those covered with IDO-expressing and non-IDO-expressing FPCG (* $p<0.001$) at day 8 post wounding. A cross-sectional micrograph (H&E) of the wound covered with either collagen alone (**Panel C**) or FPCG in absence (**Panel D**) or presence (**Panel E**) of polymer-conjugated IFN-γ is shown at day 8 post wounding (40X magnifications). Arrows indicate lateral extent of the wound. IDO immunostained cross-section of the wound treated with polymer-conjugated IFN-γ is also shown at day 8 post wounding (100X magnification) (**Panel F**).

A


M
1
2
3
IFN-γ

B

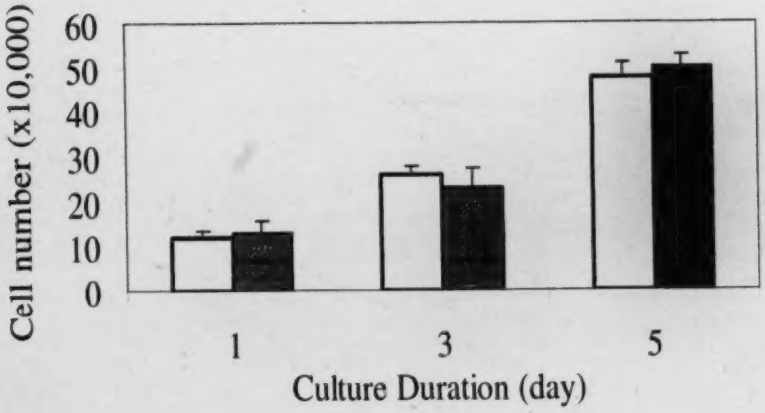
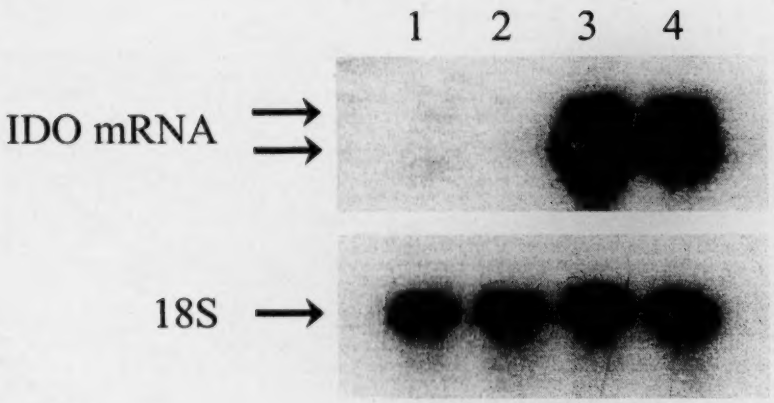

Cell number (x10,000)
60
50
40
30
20
10
0
1
3
5
Culture Duration (day)

Figure 1.5

A



B

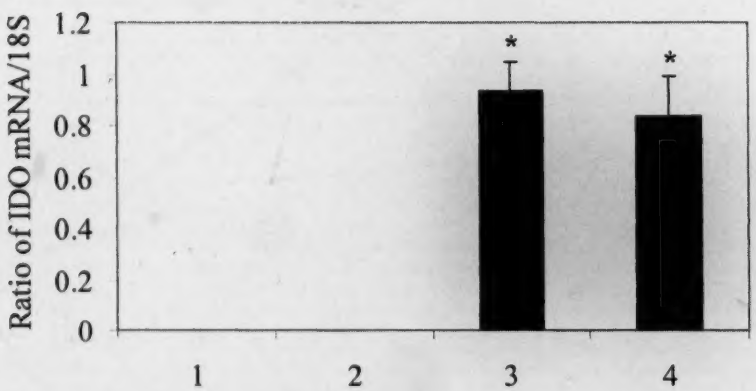
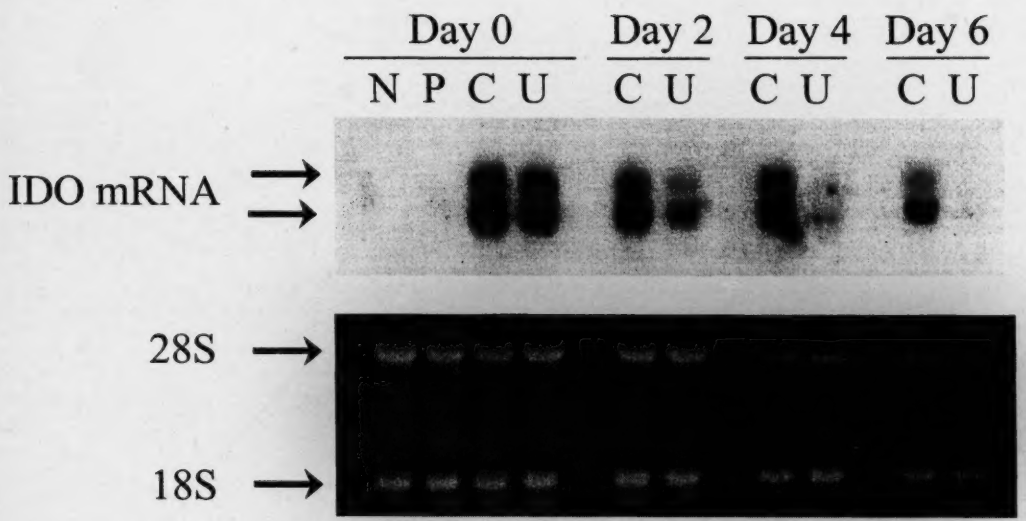


Figure 2.5

A


Day 0
Day 2
Day 4
Day 6
N P C U
C U
C U
C U
IDO mRNA
28S
18S

B

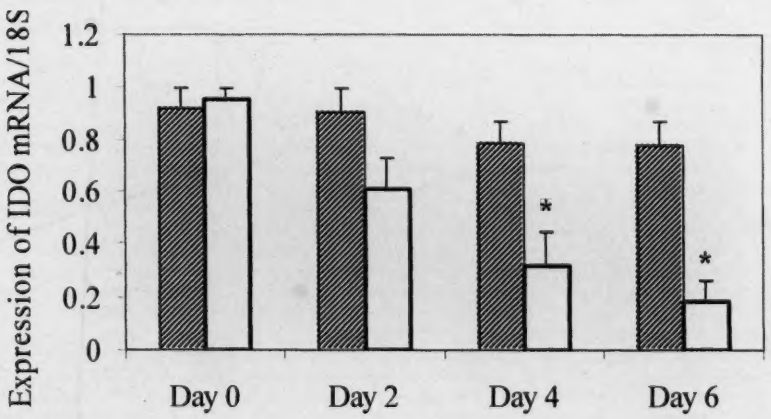
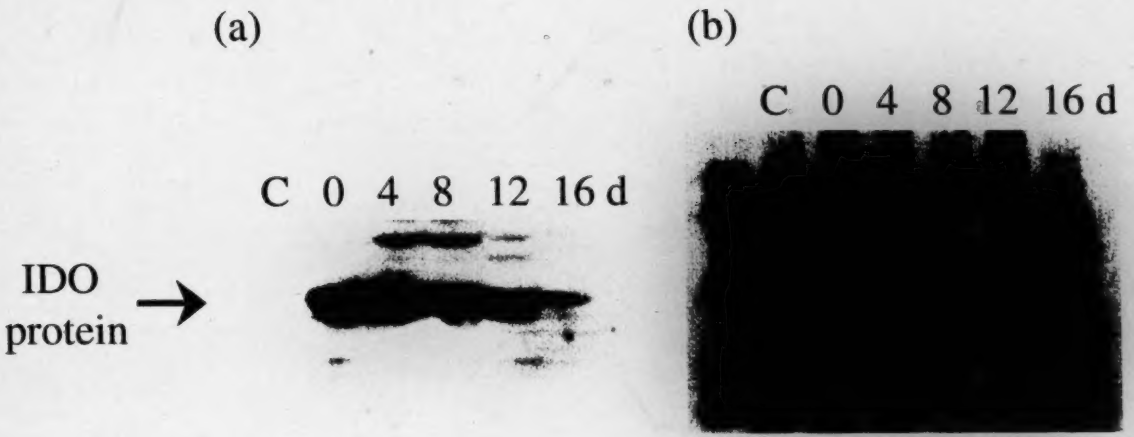

Expression of IDO mRNA/18S
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Day 0
Day 2
Day 4
Day 6

Figure 3.5

C


(a)
(b)
C 0 4 8 12 16 d
C 0 4 8 12 16 d
IDO
protein

D

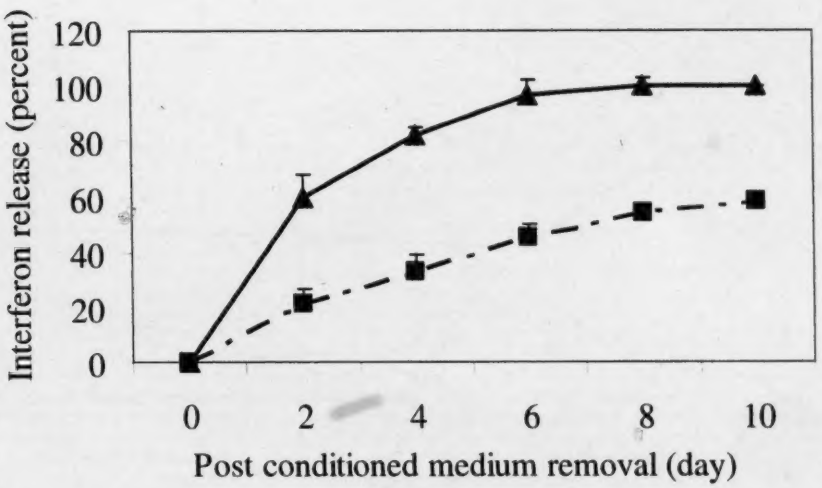
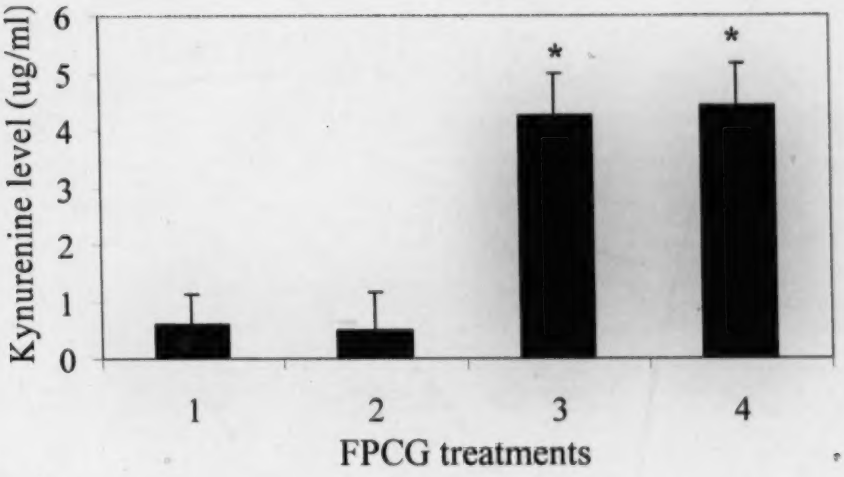

Interferon release (percent)
120
100
80
60
40
20
0
0
2
4
6
8
10
Post conditioned medium removal (day)

Figure 3.5

A


Kynurenine level (ug/ml)
0
1
2
3
4
5
6
*
*
1
2
3
4
FPCG treatments

B

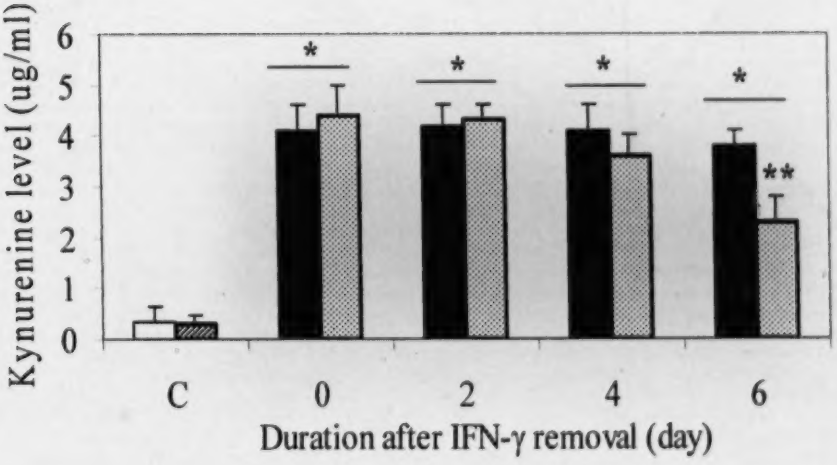

Kynurenine level (ug/ml)
0
1
2
3
4
5
6
*
*
*
*
**
C
0
2
4
6
Duration after IFN-γ removal (day)

Figure 4.5

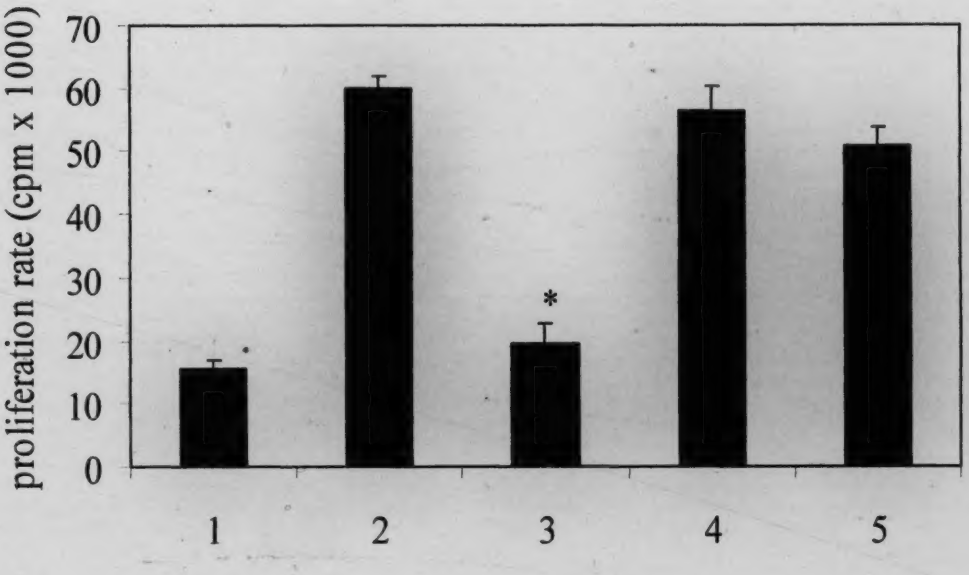
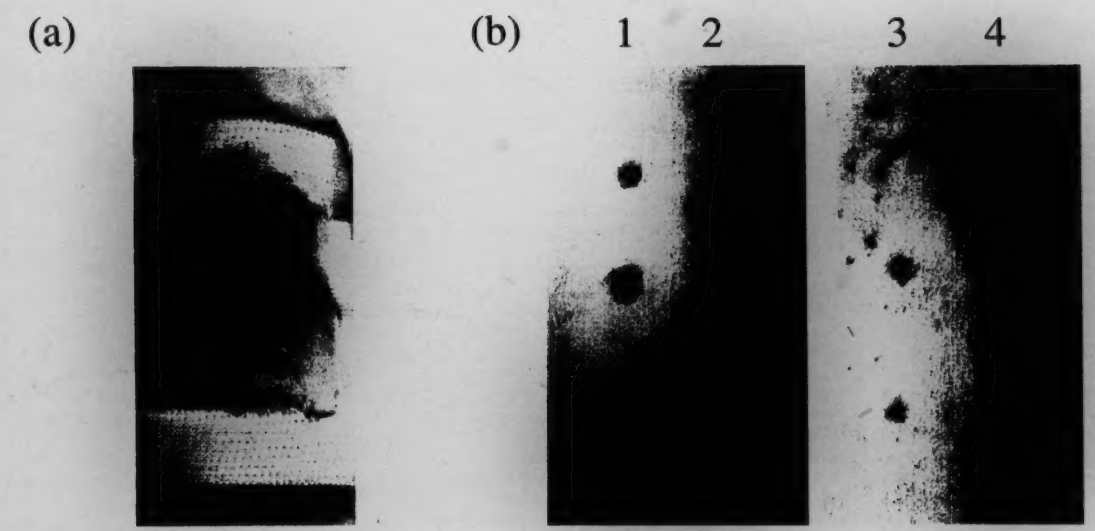


Figure 5.5

A


(a)
(b)
1
2
3
4

B

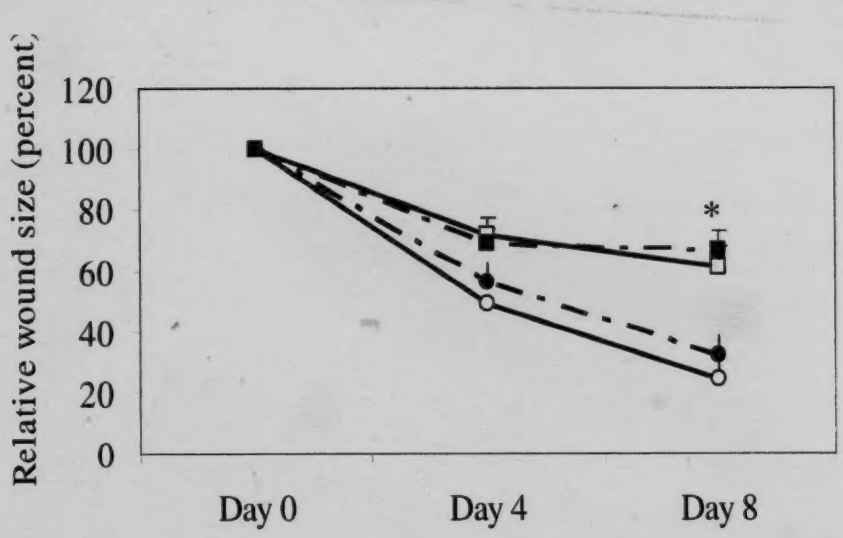
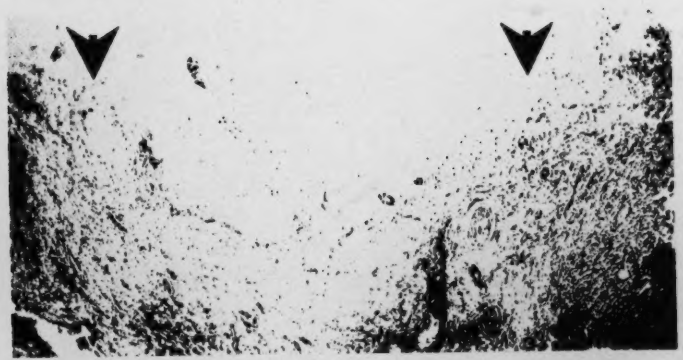

Relative wound size (percent)
120
100
80
60
40
20
0
*
Day 0
Day 4
Day 8

Figure 6.5

C



D

Figure 6.5

E

F

Figure 6.5

CHAPTER SIX:

GENERAL DISCUSSION

Discussion

The use of skin substitute for wound closure is beneficial for patients with large full-thickness burns involving a very large total body surface area (TBSA) or patients having healing problems. This artificial skin would serve as immediate wound coverage to prevent local and systemic complications resulting from burn injury and facilitate the healing. Although the procedure of Rheinwald and Green (1975) in culturing autogenic keratinocytes made coverage of extensive wounds possible, due to the absence of dermal component, its durability and engraftment were suboptimal. Furthermore, a relatively long time was required to culture and prepare the cell culture required to cover the large wounds (Jones et al, 2002). As an alternative, a bilayer skin substitute was utilized, which possessed both epidermal and dermal components (in a three dimensional cell culture system) populated with cells. However, due to the immunogenicity of these cells, the graft would become immunogenic which would further reduce the rate of the healing. Hence, to overcome the immunogenicity problem associated with these kinds of grafts, the allograft should be made non-rejectable. The purpose of our study was, therefore, to evaluate the efficacy of utilizing an immunosuppressive enzyme such as indoleamine 2,3-dioxygenase (IDO) to locally suppress proliferation of immune cells and to prolong the rate of allograft survival. Although, inhibition of immune cell proliferation due to IDO expression has been demonstrated in previous studies (Hwu et al, 2000; Logan et al, 2002), the immunosuppressive properties of IDO in dermal fibroblasts situated in a three-dimensional system (which is more similar to an *in vivo* system) has not been explored yet and therefore, needs further investigation.

As a part of series of experiments, we showed that treatment of fibroblasts grown in a three-dimensional collagen gel with IFN-γ can induce the expression of IDO mRNA in a time-and dose-dependant manner. Also, kynurenine levels, as measurements for IDO enzyme activity, were significantly higher in the IFN-γ-treated fibroblasts, compared to those in controls suggesting the biological activity of the IDO enzyme. Since an ideal skin substitute should possess both epidermal and dermal components to resemble biological and mechanical properties of the normal skin, in addition to fibroblasts, keratinocytes were also treated with IFN-γ to induce the expression of IDO. Results obtained from Northern analysis of fibroblasts and keratinocytes treated with IFN-γ for different durations of time showed that fibroblasts respond faster to IFN-γ compared to keratinocytes. Kynurenine levels were also increased at a shorter time in fibroblasts conditioned medium compared to that of keratinocytes. This might be due to differences in either rate of IDO protein translation, the environment in which the enzyme is active or both, between fibroblasts and keratinocytes. Surprisingly, the results obtained from Northern blot analysis of skin cells treated with different doses of IFN-γ showed that IDO mRNA level is expressed in keratinocytes at lower doses, compared to fibroblasts. Kynurenine levels were also significantly increased in keratinocytes at relatively lower doses of IFN-γ than in fibroblasts which suggests that keratinocytes are more sensitive to lower concentrations of IFN-γ compared to fibroblasts. This may be due to a variation in the relative abundance of IFN-γ receptors (IFN-γ R) on the surface of skin fibroblasts and keratinocytes.

The lasting effect results of IFN-γ on the expression of IDO mRNA in dermal fibroblasts revealed that IFN-γ-induced IDO expression in treated cells is transient as it is

decreased following IFN-γ removal. In fact, the level of IDO mRNA expression in treated cells returned to undetectable levels within 2 days upon IFN-γ removal. IDO protein, however, was present in fibroblasts for a significantly longer time (more than 6 days) than IDO mRNA. This finding suggests a higher protein stability of IDO and that it can be stored within cells for a longer period of time. However, the lasting effect of IDO in FPCG following IFN-γ removal is relatively short. To prolong the lasting effect of IDO expression in fibroblasts populated in collagen gel following the removal of conditioned medium (containing released IFN-γ from the collagen gel), IFN-γ was delivered to the collagen gel in the conjugated-form to a temperature-sensitive polymer.

N-isopropylacrylamide (NiPAM)-based temperature-sensitive polymers are polymers that possess temperature-dependant solubility. Due to this unique characteristic, these polymers are being explored by the pharmaceutical industry for the controlled delivery of drugs (Ron et al, 1998) and in biological systems locally for the delivery of anti-thrombogenic agents on blood-contacting surfaces (Gutowska et al, 1995). Temperature-sensitive polymers possess several unique properties. They can be conjugated to proteins without the use of additional cross-linking agents and they are in a gel phase at physiological temperatures (Uludag et al, 2000), since their lower critical solution temperature (LCST: temperature for soluble ↔ insoluble transition) is typically around 30°C. Temperature-sensitive polymers have been used previously to localize therapeutic proteins at their application site and shown to be effective in both delivering the therapeutic protein locally and inducing mitogenesis (Gao et al, 2002). Based on these findings, we speculated that they would be beneficial in delivering IFN-γ locally to induce IDO expression in fibroblasts populated in collagen gel.

The results of lasting effect of polymer-conjugated IFN-γ on the expression of IDO following conditioned medium (containing released IFN-γ from the collagen gel) removal showed that the expression of IDO is significantly prolonged in both mRNA and protein levels following conjugation with the temperature sensitive polymer. In fact, the IDO mRNA expression was still detectable at day 6 following conditioned medium removal in FPCG treated with polymer-conjugated IFN-γ, whereas in was almost undetectable in those treated with free (non-conjugated) IFN-γ. This prolongation was also observable in the IDO protein level, where IDO protein was present even 16 days post removal of the conditioned medium. This sustained expression of IDO is due to the fact that polymer can retain IFN-γ within the collagen and therefore, IDO expression is induced in FPCG for longer time. However, even with the polymer-conjugated samples, some release of IFN-γ was observed, which was likely due to sparingly soluble polymer slowly dissolved in the tissue culture medium.

To evaluate the immunosuppressive properties of IDO in IFN-γ-treated fibroblasts, IDO-expressing fibroblasts were co-cultured with peripheral blood mononuclear cells (PBMC). Results showed that IDO expression significantly suppresses proliferation of PBMC in a coculture system. To show the specificity of this inhibition to IDO action, an IDO inhibitor 1-methyl-D-trytophan was added to cocultures in different concentrations and the results showed that IDO inhibitor reverses the inhibitory effects of IDO on PBMC in a dose-dependant manner. Our results coincide with those reported by Munn et al (1999), as these investigators also showed that IDO expression in antigen-presenting cells (APCs) can regulate T cell activation through tryptophan catabolism. They concluded that expression of IDO by certain antigen-

presenting cells *in vivo* would also allow them to suppress unwanted T cell responses. The suppression of immune cell proliferation is due to the fact that IDO catalyzes the conversion of an essential amino acid, tryptophan, to kynurenine (Higuchi et al, 1967; Hayaishi, 1996; Munn et al, 1998), and presence of tryptophan is required for protein synthesis, and therefore, stimulated PBMC will not be able to proliferate in a low tryptophan level environment.

IDO expression in fibroblasts generates a tryptophan-depleted microenvironment in which immune cells are unable to proliferate compromising their viability. However, a major concern is whether IFN-γ-induced IDO-expressing fibroblasts themselves are able to proliferate in the same environment. The results of fibroblasts proliferation from collagen gel and their subsequent proliferation revealed that primary cultured fibroblast are less sensitive to an IDO-induced, low-tryptophan environment. This is because the pattern of cell migration and proliferation of FPCG was comparable between IFN-γ-treated and -untreated fibroblasts. Also, in IDO adenoviral infected cells, we have shown a marked difference in the cells responsiveness to tryptophan-depletion amongst different immune cell types examined (unpublished data). For example, $CD4^+$ cells are very sensitive to low-tryptophan levels as more than 82% of them died in low-tryptophan culture conditions. However, THP-1 cells (monocytes) are significantly less sensitive in the same environment.

Although utilizing the temperature-sensitive polymer prolongs the expression of IDO in FPCG, this IDO expression is transient and decreases over time upon IFN-γ removal. However, the expression of IDO will temporarily delay the immune cell response and T cell-mediated rejection of grafts. In a recent study, Cao et al (2003) have

shown that presence of autogenic keratinocytes in the allograft would protect rejection of grafts by switching their cytokine profile from Th1 to Th2. Therefore, we speculate that IDO expression in our skin substitute would give enough time for autogenic keratinocytes to migrate over our graft and result an immune tolerance as is suggested by these authors.

In vivo results on application of our skin substitute on wounds showed that our biological skin substitute containing FPCG can facilitate the wound healing. Since wound closure process depends on migration of autogenic keratinocytes from wound surrounding, application of FPCG seems to facilitate this process. Indeed, this is in accordance with what others investigators have found. Presence of fibroblasts within collagen gel has been shown to increase keratinocytes growth in artificial skins, compared to using an acellular dermis (Krejci et al, 1991; Shakespeare et al, 1987). Our findings are similar to results reported by these authors, since application of the skin with no cells (collagen alone) didn't have a significant effect on wound closure.

We also believe that using IFN-γ will be beneficial to the burn patients. Our group demonstrated that burn patients have lower levels of IFN-γ compared to normal patients (unpublished data). Therefore, administering IFN-γ may compensate the IFN-γ deficiency seen in these patients and reduce the occurrence of fibroproliferative disorders such as keloid and hypertrophic scarring commonly seen in these patients. Moreover, in a recent study by Halloran et al (2001), it has been demonstrated that IFN-γ can prolong allograft survival by reducing the thrombosis and early necrosis of the grafts. Therefore, utilizing IFN-γ may indeed be beneficial to prolong the skin allograft survival, which is in accordance to our recent findings. We have recently shown that the expression of IDO in transfected keratinocytes treated with IFN-γ can downregulate the expression of MHC-

I in these cells (unpublished data). Therefore, despite the known functions of IFN-γ in upregulating MHC-I expression (Boehm et al, 1997), it might still have protective functions for the allograft.

Future Studies

In this study, the expression of IDO mRNA and protein were shown to be inducible in both fibroblasts and keratinocytes *in vitro*. Also the ability of this IDO expression in suppressing proliferation of PBMC was evaluated. Although *in vitro* reduction of immune cell proliferation was observed in a coculture system of PBMC with IDO-expressing fibroblasts, an alternative approach of studying allograft survival *in vivo* in a longer time period in presence of immunoprotective enzyme (IDO) will be more practical and would shed light towards functions of IDO in protecting allografts. This study can be performed by embedding allogeneic fibroblasts within collagen gel and placing them into rat kidney capsules. This model will allow studying the rate of allogeneic immune cell infiltration in a considerably longer time than the duration utilized in our study. In addition, the effect of utilizing our biological skin substitute of fibroblasts populated in the collagen gel (FOCG) was evaluated in our *in vivo* rat model. However, it is well known that keratinocytes and endothelial cells residing in skin are more immunogeneic than fibroblasts. Therefore, application of an IDO-expressing skin substitute made up of fibroblasts and endothelial cells embedded within collagen gel with an overlaying layer of keratinocytes would better illicit the immunosuppressive functions of IDO and therefore, be a better approach. In this case, immunoprotective functions of IDO can be evaluated in presence of cells that are known to be potent in activating

immune cell-induced rejection. And finally, in order to better study the closure of wounds treated with IDO-expressing skin substitute composed of fibroblasts and keratinocytes, it is possible to create relatively larger wounds than what we used which require longer time to close. By doing so, the study would take place in a relatively longer duration which might allow infiltration of allogeneic immune cells and yield a more comprehensive study in terms of measuring both the wound closure and number of infiltrating immune cells.

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